EFFICIENT METHODS AND APPARATUS FOR HIGH-THROUGHPUT PROCESSING OF GENE SEQUENCE DATA

This application claims benefit of the priority of U.S. Provisional Application Serial No. 60/274,686 filed March 8, 2001.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates generally to the processing of gene sequence data with use of a computer, and more particularly to efficient high-throughput processing of gene sequence data to obtain reliable single nucleotide polymorphism (SNP) data and haplotype data.

2. Description of the Related Art

Bioinformatics is a field in which genes are analyzed with the use of software. A gene is an ordered sequence of nucleotides that is located at a particular position on a particular chromosome and encodes a specific functional product. A gene could be several thousand nucleotide base pairs long and, although 99% of the sequences are identical between people, forces of nature continuously pressure the DNA to change.

From generation to generation, systematic processes tend to create genetic equilibria while genetic sampling or dispersive forces create genetic diversity. Through these forces, a variant or unusual change can become not so unusual -- it will eventually find some equilibrium frequency in that population. This is a function of natural selection pressures, random genetic drift, and other variables. Over the course of time, this process happens many times and primary groups having a certain polymorphism

(or "harmless" mutation) can give rise to secondary groups that have this polymorphism, and tertiary, and so on. Such a polymorphism may be referred to as a single nucleotide polymorphism or "SNP" (pronounced "snip"). Among individuals of different groups, the gene sequence of several thousand nucleotide base pairs long could be different at 5 or 10 positions, not just one.

Founder effects have had a strong influence on our modern day population structure. Since systematic processes, such as mutation and genetic drift, occur more frequently per generation than dispersive process, such as recombination, the combinations of polymorphisms in the gene sequence are fewer than what one would expect from random distributions of the polymorphic sequence among individuals. That is, gene sequence variants are not random distributions but are rather clustered into "haplotypes," which are strings of polymorphism that describe a multi-component variant of a given gene.

To illustrate, assume there are 10 positions of variation in a gene that is 2000 nucleotide bases long in a certain limited human population. The nucleotide base identifier letters (e.g., G, C, A, and T) can be read and analyzed, and given a "0" for a normal or common letter at the position and a "1" for an abnormal or uncommon letter. If this is done for ten people, for example, the following strings of sequence for the polymorphic positions might be obtained:

Person 1:	1000100000
Person 2:	0000000000
Person 3:	1000100000
Person 4:	1111100000
Person 5:	0000000000

Person 6:	0000000000
Person 7:	1000100000
Person 8:	1000100000
Person 9:	0100000001
Person 10:	1000100100

This list is typical of that which would be found in nature. As shown above, the "1000100000" haplotype is present four times out of ten, the "0000000000" haplotype is present three times out of ten, and the "1000100100" haplotype is present one time out of ten. If this analysis is done for a large enough population, one could define all of the haplotypes in the population. The numbers would be far fewer than that expected from a multinominal probability distribution of allele combinations.

The field of bioinformatics has played an important role in the analysis and understanding of genes. The human genome database, for example, has many files of very long sequences that together constitute (at least a rough draft of) the human genome. This database was constructed from five donors and is rich in a horizontal sense from base one to base one billion. Unfortunately, however, little can be learned from this data about how people genetically differ from one another. Although some public or private databases contain gene sequence data from many different donors or even contain certain polymorphism data, these polymorphism data are unreliable. Such polymorphism data may identify SNPs that are not even SNPs at all, which may be due to the initial use of unreliable data and/or the lack of proper qualification of such data.

In order to discover new SNPs in genes, one must sequence DNA from hundreds of individuals for each of these genes. Typically, a sequence for a given person is about

500 letters long. By comparing the sequences from many different people, DNA base differences can be noticed in about 0.1% - 1.0% of the positions, and these represent candidate SNPs that can be used in screens whose role is to determine the relationship between traits and gene "flavors" in the population. The technical problem inherent to this process of discovery is that more than 1.0% of the letters are different between people in actual experiments because of sequencing artifacts, unreliable data (caused by limitations in the sequencing chemistry, namely that the quality goes down as the sequence gets longer) or software errors.

For example, if the error rate is 3% and 500 people with 500 bases of sequence each are being screened, there are (0.03)(500) = 15 sites of variation within the sequence. If the average frequency of each variant is 5%, and 500 people are being screened, there are (0.05)(0.03)(500)(500) = 375 sequence discrepancies in the data set which represent letters that are potentially different in one person from other people. Finding the "good ones" or true SNPs in these 375 letters is a daunting task because each of them must be visually inspected for quality, or subject to software that measures this quality inefficiently.

Furthermore, one must first amplify regions of the human genome from many different people before comparing the sequences to one another. To amplify these regions, a map of a gene is drawn and addresses around the regions of the gene are isolated so that the parts of the gene can be read. These regions of the gene may be referred to as coding sequences and the addresses around these regions may be referred to as primer sequences. More specifically, a primer is a single-stranded oligonucleotide

that binds, via complementary pairing, to DNA or RNA single-stranded molecules and serves for the priming of polymerases working on both DNA and RNA.

Conventional primer design programs that identify primer sequences have existed for years, but they are not suitable for efficient high-throughput data processing of genomic (very large) sequence data. Some examples of conventional primer design programs are Lasergene available from DNAStar Inc. and GenoMax available from Informax, Inc. Basically, conventional primer design programs pick the best primer pairs within a given sequence and provide many alternates from which the user selects to accomplish a particular objective.

Efficient high-throughput reliable methods are becoming critical for quickly obtaining and analyzing large amounts of genetic information for the development of new treatments and medicines. However, the conventional primer design programs are not equipped for high-throughput processing. For example, they cannot efficiently handle large sequences of data having multiple regions of interest and require a manual separation of larger design tasks into their component tasks. Such a manual method would be very time consuming for multiple regions of interest in one large sequence. The output data from these programs are also insufficient, as they bear a loose association to the actual positions provided with the input sequence. Finally, although it is important to obtain a large amount of data for accurate assessment, it is relatively expensive to perform amplification over several runs for a large number of sequences. In other words, one large amplification is less expensive to run than several smaller ones covering the same genetic region. Because there are constraints on the upper size

limit, several economic and technical variables should be considered when designing such an experiment.

Accordingly, what are needed are methods and apparatus for use in efficient high-throughput processing of gene sequence data for obtaining reliable high-quality SNP and hapolotype data.

SUMMARY OF THE INVENTION

The present invention relates generally to the processing of gene sequence data with a computer, and more particularly to efficient high-throughput processing of gene sequence data for obtaining reliable single nucleotide polymorphism (SNP) data and haplotype data. One novel software-based method involves the use of special primer selection rules which operate on lengthy gene sequences, where each sequence has a plurality of coding regions located therein. Such a sequence may have, for example, 100,000 nucleotide bases and 20 identified coding regions.

The primer selection rules may include a rule specifying that all primer pairs associated with the plurality of coding regions be obtained for a single predetermined annealing temperature. This rule could allow for the subsequent simultaneous amplification of many sequences in a single amplification run at the same annealing temperature. The rule that provides for this advantageous specification requires that each primer sequence has a length that falls within one or more limited ranges of acceptable lengths, and that each primer has a similar G+C nucleotide base pair content. The primer selection rules may also include a rule specifying that a single primer pair

be identified for two or more coding regions if they are sufficiently close together. This rule also provides for efficiency as the single primer pair may be used for the amplification of two or more coding sequences. Yet even another rule specifies that no primer sequence be selected for that which exists in prestored gene family data. This rule is important since it avoids identifying primer pairs that may amplify sequences other than those desired.

The method includes the particular acts of reading gene sequence data corresponding to the gene sequence and coding sequence data corresponding to the plurality of coding sequences within the gene sequence; identifying and storing, by following the special primer selection rules, primer pair data within the gene sequence data for one of the coding sequences; repeating the acts of identifying and storing such that primer pair data are obtained for each sequence of the plurality of coding sequences; and simultaneously amplifying the plurality of coding sequences in gene sequences from three or more individuals at the predetermined annealing temperature using the identified pairs of primer sequences.

Reliable single nucleotide polymorphism (SNP) data and haplotype data are subsequently identified with use of these amplified sequences. More particularly, the method includes the additional steps of sequencing the plurality of amplified coding sequences to produce a plurality of nucleotide base identifier strings (which include, for example, nucleotide base identifiers represented by the letters G, A, T, and C); positionally aligning the plurality of nucleotide base identifier strings to produce a

plurality of aligned nucleotide base identifier strings; and performing a comparison amongst aligned nucleotide base identifiers at each nucleotide base position.

At each nucleotide base position where a difference amongst aligned nucleotide base identifiers exists, the method includes the additional steps of reading nucleotide base quality information (for example, phred values) associated with the aligned nucleotide base identifiers where the difference exists; comparing the nucleotide base quality information with predetermined qualification data; visually displaying the nucleotide base quality information for acceptance or rejection; and if the nucleotide base quality information meets the predetermined qualification data and is accepted, providing and storing resulting data (SNP identification data) that identifies where the difference amongst the aligned base identifiers exists.

After providing and storing all of the resulting data that identifies where the differences exist, the method involves the following additional acts. For each aligned nucleotide base identifier at each nucleotide base position where a difference exists, the method involves the acts of comparing the nucleotide base identifier with a prestored nucleotide base identifier to identify whether the nucleotide base identifier is a variant; and providing and storing additional resulting data that identifies whether the nucleotide base identifier is a variant. The providing and storing of such additional resulting data may involve providing and storing a binary value of '0' for those nucleotide base identifiers that are identified as variants and a binary value of '1' for those nucleotide base identifiers that are not. The accumulated additional resulting data identifies is haplotype identification data.

Advantageously, the methods described herein allow for high-throughput processing of gene sequence data that is quick, efficient, and provides for reliable output data.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a block diagram of a computer system which embodies the present invention;

FIG. 2 is an illustration of software components which may embody or be used to implement the present invention; and

FIGs. 3A-3C form a flowchart describing a method of efficient high-throughput processing of gene sequence data.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

FIG. 1 is a block diagram of a computer system 100 which embodies the present invention. Computer system 100 includes a network 102 and computer networks 104 and 106. Network 102 is publicly accessible, and a server 108 and a database 110 which are coupled to network 102 are also publicly accessible. On the other hand, computer networks 104 and 106 are private. Each one of computer networks 104 and 106 include one or more computing devices and databases. For example, computer network 104 includes a computing device 112 and a database 114, and computer network 106 includes a computing device 116 and a database 118. The computing devices may include any suitable computing device, such as a personal computer (PC).

Network 102 may be the Internet, where an Internet Service Provider (ISP) is utilized for access to server 108 and database 110. Database 110 stores public domain gene sequence data. Also, the inventive software is preferably used in connection with and executed on computing device 112 of private network 104. Although a preferred computer system is shown and described in relation to FIG. 1, variations are not only possible, but numerous as one skilled in the art would readily understand. For example, in an alternative embodiment, network 102 may be an Intranet and database 110 a proprietary, private DNA sequence database.

The methods described herein may be embodied and implemented in connection with FIG. 1 using software components 200 shown in FIG. 2. The software may be embedded in or stored on a disk 202 or memory 204, and executable within a computer 206 or a processor 208. Thus, the inventive features may exist in a signal-bearing medium which embodies a program of machine-readable instructions executable by a processing apparatus which perform the methods.

Such software is preferably used in connection with and executed on computing device 112 of private network 104. Preferably, the system functions within the context of a PC network with a central Sun Enterprise server. The program can be loaded and run on any desktop PC that operates using the Linux or Unix operating system. Other versions could also function in a Windows environment. Alternatively, the software could operate on a publicly accessible server and available for use through a public network such as the Internet.

FIGs. 3A-3C form a flowchart which describes a method for efficient high-throughput processing of gene sequence data. This flowchart can be used in connection with software components 200 of FIG. 2 in the systems described in FIG. 1. Beginning at a start block 302 of FIG. 3A, gene sequence data corresponding to a gene sequence and coding sequence data corresponding to a plurality of coding sequences within the gene sequence are read (step 304). Next, primer pair data within the gene sequence data are identified for one of the coding sequences by following a set of primer selection rules (step 306). The set of primer selection rules includes special rules for efficient, high-throughput processing.

For example, the primer selection rules may include a rule specifying that all primer pair data for the plurality of coding regions be obtained for a single predetermined annealing temperature (e.g., 62° Celsius). This rule allows for the subsequent simultaneous amplification of many sequences in a single amplification run at the predetermined annealing temperature. This primer selection rule further specifies that each primer sequence have a length that falls within one or more limited ranges of acceptable lengths. The primer selection rules may also include a rule specifying that a single primer pair be identified for two or more coding regions if they are sufficiently close together, which provides for efficiency as the single primer pair can be used for the amplification of two or more coding sequences. As yet another example, the primer selection rules may include a rule specifying that no primer sequence data be selected for that which exists in prestored gene family data, which is

important since the program avoids selecting primer pairs that amplify sequences other than those intended.

Referring back to FIG. 3A, the primer pair data that were identified in step 306 are stored in association with the coding sequence (step 308), and may be displayed or outputted. If additional coding sequences need to be considered (step 310), the next coding sequence is selected (step 312) and steps 306 and 308 are repeated. Thus, the acts of identifying and storing are repeated such that primer pair data are obtained for each coding sequence within the gene sequence. Once all of the coding sequences have been considered at step 310, the primer sequences are used in the amplification process.

In particular, the plurality of coding sequences in gene sequences from three or more individuals (typically 100s of individuals) are simultaneously amplified in a gene amplification machine at the predetermined annealing temperature using the identified pairs of primer sequences (step 314). In the embodiment described, the predetermined annealing temperature is 62° Celsius, but in practice it may be any suitable temperature. Next, the plurality of amplified coding sequences are sequenced to produce a plurality of nucleotide base identifier strings (step 316). Each nucleotide base identifier string corresponds to a respective sequence of the plurality of amplified coding sequences. In the embodiment described, the nucleotide base identifiers are represented by the letters G, A, T, and C. The partial flowchart of FIG. 3A ends at a connector B 318, which connects with connector B 318 of FIG. 3B.

Single nucleotide polymorphism (SNP) data and haplotype data are subsequently identified with use of these amplified sequences. Beginning at connector

B 318 of FIG. 3B, each string of the plurality of nucleotide base identifier strings is positionally aligned with the other to produce a plurality of aligned nucleotide base identifier strings (step 320). This may be performed with use of conventional Clustal functionality, which is described later below. Next, a comparison amongst aligned nucleotide base identifiers is performed at a given nucleotide base position (step 322).

If a difference amongst aligned nucleotide base identifiers exists (step 324), nucleotide base quality information associated with the aligned nucleotide base identifiers where the difference exists is read (step 326). This nucelotide base quality information may be, for example, phred values described later below. The nucleotide base quality information is then compared with predetermined qualification data (step 328). Next, the nucleotide base quality information is visually displayed for acceptance or rejection by the end-user (step 330). This step is important because phred values in themselves are not entirely adequate for determining quality. The reason is that phred uses a relative signal-to-noise ratio, but common sequence artifacts often show as signals having high ratios. If the nucleotide base quality information meets the predetermined qualification data and is accepted (step 332), resulting data (SNP identification data) that identifies where the difference amongst the aligned base identifiers exists is provided (step 334). This resulting data is stored (step 336).

If there are additional nucleotide base positions (step 338), the next nucleotide base position is considered (step 340) and steps 322-338 are repeated. Thus, steps 322-338 continue to execute until all of the differences amongst the aligned nucleotide base identifiers are identified. Step 338 is also executed if no difference exists at step 324, if

the nucleotide base quality information is not acceptable at step 332, or if the user rejects the finding based on its visual appearance. The partial flowchart of FIG. 3B ends at a connector C 342, which connects with connector C 342 in FIG. 3C.

After providing and storing all resulting data that identify where differences amongst the aligned nucleotide base identifiers exist, additional acts are performed starting at connector C 342 of FIG. 3C. At a nucleotide base position where a difference exists, the nucleotide base identifier is compared with a prestored nucleotide base identifier in order to identify whether it is a variant (step 344). The prestored nucleotide base identifier is known from the stored data in step 336. This data could be stored as variant nucleotide bases or as encoded sites (for example major, minor).

Next, additional resulting data that identifies whether a given nucleotide base identifier is a variant is provided (step 348). This additional resulting data is stored (step 350) and may be displayed or outputted. Where differences do not exist amongst aligned nucleotide base identifiers, it is assumed that no variants exist. Steps 348-350 may involve providing and storing a binary value of '0' for those nucleotide base identifiers that are identified as variants, and a binary value of '1' for those nucleotide base identifiers that are not. If additional nucleotide base positions need to be considered (step 352), then the next nucleotide base position is selected (step 354) and steps 344-352 are repeated. Step 352 is also executed if no difference is found at step 346. Thus, repeating of the acts occurs so that they are performed for each aligned nucleotide base identifier at each nucleotide base position where a difference exists. The repeating of steps ends when all nucleotide base positions have been considered at

step 352. The combined additional resulting data provide haplotype identification data (step 356).

Additional Details Regarding Primer Sequence Selection and Amplification. Regarding steps 302-314 in FIG. 3A above, which may be referred to as the preamplification process, raw human genome data is used and the method basically draws little maps with the data. Additional details regarding the preamplification process will now be described.

Coding sequences are regions within a gene sequence that encode the protein of a gene. RNA is made from DNA only at these positions. When the RNA is turned into protein, the protein sequence is a translation of the DNA sequence at the coding region. The sequence between coding sequences is called intron, which is a DNA section that divides exons. Exons are the DNA segments that store information about the part of the amino acid sequence of the protein.

The object of the present invention is to survey the coding sequences at each coding region for a given gene in many different people, which is time consuming and expensive using conventional approaches. Therefore, a preamplification strategy is designed so that many sequences can be read in an efficient and inexpensive manner. Amplification uses two addresses, one in front of the region of interest and one behind it. These two addresses define sites where short pieces of DNA bind and are extended by an enzyme called thermus aquaticus (TAQ) polymerease. Preferably, a high fidelity TAQ variant would be used, such as Pfu polymerase. The two pieces of DNA together with the enzyme result in the amplification or geometric increase in the copy number of

the sequence between the two addresses. After amplification, the software processes read and compare many sequences to one another to find out where people differ. Without amplification, there is too little DNA to read.

One object of the preamplification process is to appropriately select these addresses, which are the primer sequences, for each one of the coding regions. Ordinarily, this is not a trivial task. For any given coding region, there are typically large numbers of potential primer pair solutions from which to select, and often most of these would result in an inefficient or failed amplification because of non-specificity. The preamplification process described herein works in connection with a plurality of coding regions for many genes and identifies a plurality of primer regions so that amplification can be performed in a specific, cost-effective, and efficient manner.

The software program accepts as input: (1) a genome database sequence file, which may be many hundreds of thousands of letters long and downloaded from the freely available human genome database (default format for convenience); (2) data (e.g., numbers) that indicate where the coding regions are in the input sequence file. The file containing the coding region data (taken from the annotation of a publicly accessible human genome data file) may be referred to as a "join" file because the data in this file typically resemble the following:

```
join(8982..9313, 1..81, 17131..17389, 20010..20169, 21754..22353)/gene="CES1 AC020766"
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OR

```
join(81..140,1149..1320,1827..2092,2402..2548,2648..3089)/gene="example gene AC10003"
```

In the second-listed join file above, the first coding region indicated is the region from 81 to 140; the second coding region indicated is from 1149 to 1320, etc. The object is to select a small region of sequence (e.g., 18-22 letters) in front of and behind each coding region in the input sequence file for each coding region identified in the join file. These small sequences are the primers and, for each identified coding region, the program finds a flanking pair of primer sequences. These primer sequences are then named and presented to the user.

Using the two input files, the software is designed to more particularly perform the following in association with steps 302-314 of FIG. 3A:

(1) Use the numbers in the input join file to identify the coding regions in the input sequence file;

(2) Identify or select suitable primer regions around coding regions in the most efficient manner (e.g., sometimes the primers will flank a single coding region, and sometimes they will flank two or even three coding regions if they are close enough to be amplified efficiently);

(3) Select primer pairs for the same annealing temperature (i.e., the temperature required to get them to do their job during amplification). Thus, if one designs ten primer pairs all with the same annealing temperature, say 62° Celsius, they can all be used in an amplification machine together as each amplification run uses a single fixed temperature;

(4) Avoid ambiguous letters (e.g. the letter "n") when selecting primer regions;

(5) Design primers using a strategy to reduce the chance that the primer will be within what is called a "repeat" region. This strategy involves recognizing poly-A stretches, ensuring that the least amount of intron sequence possible is present between the two primers (as repeats tend to be removed from exon boundaries by buffer space);

(6) Display to the user all of the statistics surrounding the selections (as examples, how many letters exist between two primers of a pair, the precise numerical position of each of the selected primers, etc.); and

(7) Output the primer sequences in a database compatible format (e.g., tab delimited) for easy ordering from primer synthesis vendors.

Now the following input join file

```
join (81..140)/ gene="example gene AC10009"
```

and the following input sequence file

```
  1 GAATTCTTTC CAGAAGGCTT TCCATTTACT TTTCCTAGAT TCATCAGAAG AATCATTATC
 61 TACAGCAGCT GTAACTGATT GAAATGTATT TTATGAACAA TAAGACTTGA AAGTTAAAAT
121 TGCTCCTTTA TCCATGTACT GAAGAATAAA TATTGTGAAA GCAGTCATAA AAACAGAAGT
181 AATCTTTTGG TACCTCTGCA TTAGAACTCT TTATTAACCA GGTGTATTGC CATTCAACAG
241 TAATATTTTG AAAGGAATCT CTATTTTTGA GCAGGTTTCA ACTTCTGCTT TTTATTTTAA
301 ACAGTAGACT TGAAATATTC AGTAACCATG CTATAAAGAG CTATGCTGTA AGACAGCTTT
361 TTCTATTTAT AGAGCATGGT TTTGAAATTA TAACAAAGCA TGGGTTTTAT CCTGAAATCA
421 TTCATAAATA ACACGTACCA AAACTTTAAT ACGGGCTAGC CAGTGTGAGC CAGTGTGACG
```

are considered. For the input sequence file, the number of the first letter of a line is shown at the beginning of each line and there are spaces every ten letters. Typically, there is an annotation before the sequence in the file, such as that shown below, which is ignored by the software:

```
LOCUS       AL355303   157796 bp    DNA             HTG       08-SEP-2000
```

```
DEFINITION  Homo sapiens chromosome 10 clone RP11-445P17, *** SEQUENCING IN
            PROGRESS ***, 19 unordered pieces.
ACCESSION   AL355303
VERSION     AL355303.11  GI:10086110
KEYWORDS    HTG; HTGS_PHASE1; HTGS_DRAFT.
SOURCE      human.
```

The input join file identifies the coding region, which is underlined in the sequence below:

```
  1 GAATTCTTTC CAGAAGGCTT TCCATTTACT TTTCCTAGAT TCATCAGAAG AATCATTATC
 61 TACAGCAGCT GTAACTGATT GAAATGTATT TTATGAACAA TAAGACTTGA AAGTTAAAAT
121 TGCTCCTTTA TCCATGTACT GAAGAATAAA TATTGTGAAA GCAGTCATAA AAACAGAAGT
181 AATCTTTTGG TACCTCTGCA TTAGAACTCT TTATTAACCA GGTGTATTGC CATTCAACAG
241 TAATATTTTG AAAGGAATCT CTATTTTTGA GCAGGTTTCA ACTTCTGCTT TTTATTTTAA
301 ACAGTAGACT TGAAATATTC AGTAACCATG CTATAAAGAG CTATGCTGTA AGACAGCTTT
361 TTCTATTTAT AGAGCATGGT TTTGAAATTA TAACAAAGCA TGGGTTTTAT CCTGAAATCA
421 TTCATAAATA GCACGTACCA AGACTTGAAC ACGGGCTAGC CAGTGTGAGC CAGTGTGACG
```

Short sequences (e.g., between 18-22 letters) in front of and behind this coding region are selected based on a set of primer selection rules. The program then names these two primer sequences and presents them to the user at the end of the analysis. This is done seamlessly for multiple coding regions identified in the input join file. From the example above, the following primer pair data (in small letters) are selected or designed for the given coding region:

```
  1 GAATTCTttc cagaaggctt tccatttacT TTTCCTAGAT TCATCAGAAG AATCATTATC
 61 TACAGCAGCT GTAACTGATT GAAATGTATT TTATGAACAA TAAGACTTGA AAGTTAAAAT
121 TGCTCCTTTA TCCATGTACT GAAGAATAAA TATTGTGAAA GCAGTCATAA AAACAGAAGT
181 AATCTTTTGG TACCTCTGCA TTAGAACTCT TTATTAACCA GGTGTATTGC CATTCAACAG
241 TAATATTTTG AAAGGAATCT CTATTTTTGA GCAGGTTTCA ACTTCTGCTT TTTATTTTAA
301 ACAGTAGACT TGAAATATTC AGTAACCATG CTATAAAGAG CTATGCTGTA AGACAGCTTT
361 TTCTATTTAT AGAGCATGGT TTTGAAATTA TAACAAAGCA TGGGTTTTAT CCTGAAATCA
421 TTCATAAATa gcacgtacca agacttgaac ACGGGCTAGC CAGTGTGAGC CAGTGTGACG
```

Since there are typically about ten important regions in a given sequence, there are typically about twenty short primer sequences which are produced. Oftentimes, however, a single primer pair that flanks two (or more) coding regions is picked so that the actual total number of identified primer pairs will be less than two times the number of coding regions.

The two sequences are also named according to specific rules. Here, the names for the example as TPMTE2-5 and TPMTE2-3 are given. The two primer sequences are presented to the user in the output form below.

```
TPMTE2-5 ttccagaaggctttccatttac
TPMTE2-3 gttcaagtcttggtacgtgct
```

Note that the TPMTE2-5 sequence is identical to the first picked sequence whereas the second sequence, TPMTE2-3, is the reverse and compliment of the second picked sequence.

In the preferred embodiment, the following set of primer selection rules are used for selecting primer sequences:

Rule 1: The number of combined "G"s and "C"s should be roughly equal the number of combined "A"s and "T"s.

Rule 2: There should be no longer than four consecutive "G"s together (e.g., ...GGGG...), four consecutive "C"s together, four consecutive "A"s together, and four consecutive "T"s together.

Rule 3: The length of each primer sequence should fall within the range of 18-22 (inclusive). The length is determined by giving a value of four for each "G", four for each "C", two for an "A", and two for a "T", and then calculating the sum of numbers such that the total sum for any selected sequence must equal 62. Thus, depending on the number of "G"s, "C"s, "T"s and "A"s, the total length of sequence necessary to get a value of 62 will

usually fall within the range of 18 to 22 letters (inclusive).

Rule 4: The number of letters that fall in between the two selected sequences (herein referred to as a "block") should be equal to some rough integer multiple of 420 letters. For example, the number can be 420, 840, 1280, 1700, or 2120 (2120 is the maximum and 420 is the minimum). The number of letters does not need to be exactly 420, 840, or 1280, etc. however, but can be reasonably close; say plus or minus 50 or even 75. This range also can be chosen arbitrarily at first and then modified later. For example, if plus or minus 50 is chosen, the range should be 370-470, 790-890, or 1230-1330, etc.

Rule 5: At least one of the primer sequences must be within 100 letters of the beginning or the end of the coding region.

Rule 6: If the size of a block is larger than 1400, a third short sequence should be picked to reside roughly at position "700" in that block. This sequence should have the letters "seq" at the end of its name. For example, in the sequence below, the block is 2290 letters long:

```
   1 GAATTCTttc cagaaggctt tccatttacT TTTCCTAGAT TCATCAGAAG AATCATTATC
  61 TACAGCAGCT GTAACTGATT GAAATGTATT TTATGAACAA TAAGACTTGA AAGTTAAAAT
 121 TGCTCCTTTA TCCATGTACT GAAGAATAAA TATTGTGAAA GCAGTCATAA AAACAGAAGT
 181 AATCTTTTGG TACCTCTGCA TTAGAACTCT TTATTAACCA GGTGTATTGC CATTCAACAG
 241 TAATATTTTG AAAGGAATCT CTATTTTTGA GCAGGTTTCA ACTTCTGCTT TTTATTTTAA
 301 ACAGTAGACT TGAAATATTC AGTAACCATG CTATAAAGAG CTATGCTGTA AGACAGCTTT
 361 TTCTATTTAT AGAGCATGGT TTTGAAATTA TAACAAAGCA TGGGTTTTAT CCTGAAATCA
 421 TGCTCCTTTA TCCATGTACT GAAGAATAAA TATTGTGAAA GCAGTCATAA AAACAGAAGT
 481 AATCTTTTgg tacctctgca ttagaactcT TTATTAACCA GGTGTATTGC CATTCAACAG
 541 TAATATTTTG AAAGGAATCT CTATTTTTGA GCAGGTTTCA ACTTCTGCTT TTTATTTTAA
 601 ACAGTAGACT TGAAATATTC AGTAACCATG CTATAAAGAG CTATGCTGTA AGACAGCTTT
 661 TTCTATTTAT AGAGCATGGT TTTGAAATTA TAACAAAGCA TGGGTTTTAT CCTGAAATCA
 721 TGctcctttg tccatgtact gaagAATAAA TATTGTGAAA GCAGTCATAA AAACAGAAGT
…1000 bases …
1781 AATCTTTTGG TACCTCTGCA TTAGAACTCT TTATTAACCA GGTGTATTGC CATTCAACAG
1841 TAATATTTTG AAAGGAATCT CTATTTTTGA GCAGGTTTCA ACTTCTGCTT TTTATTTTAA
1901 ACAGTAGACT TGAAATATTC AGTAACCATG CTATAAAGAG CTATGCTGTA AGACAGCTTT
1961 TTCTATTTAT AGAGCATGGT TTTGAAATTA TAACAAAGCA TGGGTTTTAT CCTGAAATCA
2021 TGCTCCTTTA TCCATGTACT GAAGAATAAA TATTGTGAAA GCAGTCATAA AAACAGAAGT
2081 AATCTTTTGG TACCTCTGCA TTAGAACTCT TTATTAACCA GGTGTATTGC CATTCAACAG
2141 TAATATTTTG AAAGGAATCT CTATTTTTGA GCAGGTTTCA ACTTCTGCTT TTTATTTTAA
2201 ACAGTAGACT TGAAATATTC AGTAACCATG CTATAAAGAG CTATGCTGTA AGACAGCTTT
2261 TTCATAAATa gcacgtacca agacttgaac
```

At the region around the letter at position "700", one cannot find a third short sequence that meets the criteria of having roughly equal G+C and A+T. A suitable sequence

around position "723", however, can be found and is shown in lower case. In this example, three sequences are presented to the user: the first two read exactly as they appear in the lower case letters, and the last one being a reverse and compliment of the sequence at position "2270":

```
TPMTE2-5 ttccagaaggctttccatttac
TPMTE2-seq ggtacctctgcattagaactc
TPMTE2-3 gttcaagtcttggtacgtgct
```

The following is a logic summary for the primer identification rules according to the preferred embodiment:

(1) Define the smallest block of sequence that surrounds and completely encompasses the coding region and is either 700 (+/-100) letters long, 1400 (+/- 100) letters long, 2100 (+/-100) letters long, 2800 letters long (+/-200). That is, identify the smallest such block from those having a length = n*(700 +/- 100) for n = {1, 2, 3, 4}.

(2) Find a sequence at the beginning of this block such that:

(a) the sequence is 18-22 letters long;

(b) the value of the sum of the letters is exactly 62, where a G=4, C=4, A=2 and T=2. Put another way, Sum (T) *2 + Sum (A)*2 + Sum (G)*4 + Sum (C)*4 = 62. Using this rule, G+C will be either 9, 10, or 11 since only with these values is it possible to have a sequence that is 18-22 letters long with the sum of values = 64;

(c) No greater than four of the same consecutive letters must exist (e.g., …TTT… is fine but …GGGGG… is not) and, if a string of four letters exist in the "5" prime primer, the same string of four or three letters should not exist in the "3" prime primer; and

(d) the last letter should be a "G" or a "C", not an "A" or a "T".

(3) Find a sequence following the end of the block such that the sequence follows the same rules as described in (2) above.

(4) After identifying two or more blocks, if two blocks can be constructed in the input sequence such that the end of one block overlaps with the beginning of another, or such that the end of one is within, say 100 letters of the beginning of another, the two blocks are merged, as long as the new merged block is not greater than 2800 (+/-200). It is preferable to have one large block compared to two or more smaller ones. If the blocks are merged, the first sequence selected for the first block and the last sequence selected for the second block forms the two sequences of the new merged block. The second sequence for the first block and the first sequence of the second block are discarded.

The selected sequences are also named by the software, preferably as follows. There are three parts to the name. The first is the gene which is the same as the input sequence file name. For example, for the gene "TPMT" all sequences the program finds for the input sequence file will have "TPMT" in the name. In addition, the first block found includes in its name "E1", the second block found includes in its name "E2", the third "E3", and so on. If two blocks are merged, however, both of these tags will be included in the name of the merged block in order. For example, if "E1" and "E2" blocks are merged, then the characters "E1E2" will be in the new name for the new merged block. Finally, the first sequence found for a block will have the characters "-5" and the second will have the characters "-3".

Below is a naming example where there are five blocks and two sequences for each block, except where blocks "2" and "3" were merged, and the merged block is 1260 (+/-100) letters long and required a third sequence to be selected:

```
TPMTE1-5
TPMTE1-3

TPMTE2E3-5
TPMTE2E3-3
```

TPMTE2E3SEQ

TPMTE4-5
TPMTE4-3

TPMTE5-5
TPMTE5-3

Another way to describe the naming process is presented. The 5-prime and the 3-prime primer may be presented to the user based on the following logic:

(1) The name of the gene (which is the sequence file name) and block appears in the name of each primer sequence;
(2) The gene and block name corresponding to the sequence file is provided in front of the name for a block is provided. If the sequence file is named "AHR", for example, the first block name would include "AHRE1" and the second block name would include "AHRE2";
(3) The "5" prime or "3" prime designation is also presented in the name of the primer. For example, the primers for the first block of the AHR gene would read:

AHRE1-5 - *the first sequence found (sequence whose numerical position is least - e.g. at position 60)*

AHRE1-3 - *the second sequence found (sequence whose numerical position is most - e.g. at position 420)*

After naming, the sequence of letters for each primer sequence may be presented as follows:

1. Present the first sequence (called the "5" primer) as it appears in the sequence, letter for letter but without the blank spaces;
2. Present the second sequence (called the "3" primer) such that
   a. The sequence is reversed such that the end is now the beginning and the beginning is now the end and then,
   b. "A" is substituted for each "T"
   c. "T" is substituted for each "A"
   d. "G" is substituted for each "C"
   e. "C" is substituted for each "G"

(For example: "AATTATGCCT" would become "AGGCATAATT")

3. Present any third sequence for a block (if necessary because the block is 1260 +/- 100 letters long) as it appears in the input sequence exactly, letter for letter but without blank spaces.

An example output looks like:

```
TYRE15 TTGCATGTTGCAAATGATGTCC
TYRE13 CAACCCAGGTCATCGTTCAC

TYRE25 CCTCTCAAGCACATTGATCAC
TYRE23 TATACTGATCTGAGCTGAGGC
```

and so on, until...

```
TYRE9-5 TAACATTCACACTAATGGCAGC
TYRE9-3 TGCTTCTCCTCTAGAGGCTG
```

The numerical position of each primer sequence relative to the input sequence is preferably presented as well.

The following is an example summary of a join file, a gene sequence file (including relevant portions only for brevity), and output data, for the gene "CES1 AC020766". In the gene sequence file below, the coding regions are highlighted in bold print.

```
==========================================================================
JOIN FILE FOR GENE "CES1 AC020766"

join(80513..81472,81911..82007,82114..82219,85116..85265,89595..89651)/gene="
CES1 AC020766"
==========================================================================


==========================================================================
GENE SEQUENCE FILE FOR "CES1 AC020766"

        1 aacttagcaa acacatgatc ttgtatatag tagacatcat tattgttttc ccctctattc
       61 ttcttttcaa tttctgaatc ataaggattg cctgagccta ggagatcaag gccagccttg
      121 gcaacatggc gaaatgccat ctctacaaaa aaaaaaaaaa aaattatcta ggtgtggtgg
      181 caagcaccag tggtcccagc tactcagaag gctgaggtgg gaggattgct tgagcccagg

                                        *
                                        *
                                        *
```

28561 agtagagtgc tggcatactc agtaagacta tattgaataa atgaatgaat aaccccagaa
28621 taaaaatgta actataaatg tgttatccta ggtctcaaat cagaatgatc tgaaagttag
28681 gaaacccccc tgccactgca gagatctcat cttactttta tgtcctatta taatgggaga
28741 ctatggcaag aaatttttga tatctacaga atagatctct atttggacca attttcatct
28801 ttgtttgatt caataaacag gctaagttct acttacgaag cctataaaac tccaaaactc
28861 caaatatcca catattccta aatatgtcac ctaactctaa tacatataca acatgatgag
28921 tacacatcct gtccattttc aagaacttat gcactcatca ctgtacacct tgatatctag

*
*
*

79801 agttaatgca cacagtttgg ctagttttgg cttcaaaatt aattaaactg tatcaatgta
79861 ttttgaagtg ttaagtcatc tgtatgcttt agctccttct atagatgagg caaatataca
79921 aacagattaa actgactttt acagaataat tattctttta ccttgtttac atggaaagga
79981 atcctccatt ttaggatgca cataaaatgc cagcctatgt tgatgacatt gccttaacac
80041 tttttttta agtaatttta cagggtagtt aacctgtaaa agaaacagtg gataaacttg
80101 aaaatgctaa tagcaaaaaa cacttcagcc atggcacata caaccagaag ccaatgatat
80161 ccttcaacta tagaaattag cggtgttttc tgtttattcc tgaagcagga ttccatattc
80221 aagccagaaa ttgtcattca acagaaaaaa tcaggtcaaa acaatcaatc acataatgta
80281 gcaagacaaa agtatgtgct tatgtgaaga aaaacaaaaa caacaaataa ccgaactttt
80341 attttcttga atataatatt gatggcaaga ttgctaagag gtcatccctg tatttagttt
80401 agataaaggc ttccagcata gaacactgtt aagaagtaac tgtcaggagc tatgcagaag
80461 tgatgagagg caaataatat aaaaactaga aaagcaggtt ttaatttttct atagacttta
80521 ttacacatta ttatgttacg agacaaatgc agataattct taatttatca aatttgtgag
80581 cttaattaac aaaaatattt gaccctcacc agaaaaacag ataactctaa atctactctg
80641 aaaatctaat caattgcgaa gtattaccta tttggagact atgtattata tcaaagataa
80701 agctactatt ctcacagaac atatggggtc attggcagcc aaccaataat gaagtaaata
80761 ttctaatatt tgggaaaata ctgagaaaac taataaattg tcctggatat tatttattct
80821 tgcctttaca aaagacttac acatccaaat gagattagtt tagaatagag gtttttagtt
80881 cagaaaatgt tcaaagtcca atacagtcat ggctaatcag agactagaga acctttataa
80941 aggtaagtag gcttgaaaac ccttggaaac tgagcagtct tattttgaac tagcatgttt
81001 taatcaaagg tatggaatta atcaaatatc aattaagaat tactggaatg cacactcatg
81061 ccaaatgaca actaacatgt tatttcctac tatgatgact ctttgatttg agtcagatgg
81121 ataaaaaaaa tattgctagc tatacaataa attttactct tctgcttctg ctctctaaag
81181 aaaaatctta ttttttcaca taagaagctc atggaatcga atgttaatta aagaaaagat
81241 agggtaagta caactggggg aaagacagta cctctaatta ataggaaat ccatgaaaga
81301 attaatcatc ataagagaag aatcattttt ccagtagccc cactaccatg aatgatattt
81361 tcatgagcct cggccacctt ctccaatgga tattgagaac ctatcacagg tttcaaccag
81421 ccaatttcca ttccagcttg aagggctgct gcatattgct gaaattcctc ctaagaaaag
81481 gaaaaacaaa tttctttttg tagtgaaccg tatgatttaa ttttcagaag cattaaaaac
81541 acttcagaat ctaagtgtta taccatgaag agtctcttac aaatgtgtga cttttgtcaa
81601 cttgtccaga actatagaaa aagtagttat ctacagggta accataaatc ccatctgcct
81661 gagacagtgt tagtgtacaa aatacctgtt gtcctgaaat tattactagt atcacatttc
81721 tatctcaaaa ggtatgctta cctggatata aattatactg tcaccctagt tgtccttctg
81781 gtgactaatc cttaccaact cccactagtc atataactaa gtttaacatc tattcaaact
81841 ttcagcttgc ctgagtaggc aaactgtacc aatgtttaag ttaccaaaat cagaagtact
81901 tcttttccta ccttggttga ggaaaagaga gtaactccaa ttatactcga ctcctttgcc
81961 atggtgtctc gtgggtttat ttcaatagta cctctgctgc caacaaccta acatgaaaaa
82021 cagcaattct acagttaaag attactgtaa aatagtgtta aattgtggta aaacattaaa
82081 gtggtaaaaa aaaaaaaaag aaaaggaata cttactatca ctcgtcctcc atgtgacaga
82141 agactcaagt ctttactaag atttacatta gctaacattt caataattat atcaattcct
82201 ttctcaccaa catacttcta tataataaaa gagaaatgta gagtaagata gcaagtgaaa
82261 aactgtaaaa tagctactat ctgtacaaga tattatagaa atatgtttca aatgatatat
82321 aaatgctaca tctttgagac taataatgca aaattttaaa taatctaatt atataatcac
82381 gatgtaattc caaggtacca gccagaacat ctaaactgat aaaaatttgt actaaataca

```
82441 ttgctgtagt gaaataaagt ttgtctggaa ttttcaggtg ctagactcaa cttgagtata
82501 aaatacttag ctgaaaattt tctatctgta aaataaactt tcataaagaa acaataaatc
82561 aaaagcccca aacccccagg gggctcccat ttttattaat aaacaaaaag caaaagaaga
82621 tatcattagc tgttcggttt tgcatgattt ttgttgtttt agtgcatttg gttttgttct
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82981 tgattgcctg ccatggtcag ataaagttcc acagagacga tgaactgggc cctcagggat
83041 gaataggagt ttcccaagcc aaaagaaagg aaaatgagta aggggaagct agacctgagg
83101 ctgagtcagt ctggaccaaa gaaacagaaa agcaaagatg gaggggactg agaacacaag

                                    *
                                    *
                                    *

84301 taacgggcca tttttcatct ttgtgaatat tcttggataa tggtatcagc agtgctagat
84361 cttaggttcc ccagacgtat aacaaaggag tgcttttgtt cggctttttg gcaagatgat
84421 tgcaaaaaag gtaataaact ctcactctta ttttttcctt catttgtaat gatctaattt
84481 acacagtact caatatttgg gaaattctaa tctccccaac gtgaggaagt ggttgaggat
84541 tagcaaagca ataagtgttt agcaaattgc taatatagta caagtgaaga acttcagaat
84601 ctgcttgaat tctgttaaat gcagcaacta aataaatgcc acctcaccat tttggatgca
84661 gtagtgatta ttcctccaaa gcatccagct aacaaatgaa ctttattccc tgggccacac
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84841 ttgcattaaa aaatcaattt ttatagctct aagattctag agaagctata ttctatttaa
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84961 ataaattaaa gttttaaaac acgcataaaa attcatcgca ctgaaaaaag gaagcaaaca
85021 gctttaaagg agtagttggt taaaaacata ttaaaaaacc acgcaagtct ccaaggaaca
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85201 gtaccagcag tgcccaaaat ctttaagcca taagctctag caatttggca tgctgctaat
85261 ccaacctgaa aaacaaatat aacccaagag ttatatattc tctacactcc tgtaaacact
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85381 gagtgtaaat gaagaaataa gaaaaacagc taactttaat tgagcattta aaatattcca
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85501 agtagatact attattgtcc ctattgtaca gataagaaag ttgaagcttc aaattataag
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85681 tctctgactt ctttgtgcat atgaaattct aaataacaaa tgtaaggcct ccaccattta
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86401 aagagatgaa gctggcttga acaaaggtgg tggcactgga aaaaataaac aaattcagat
86461 atagtttaga ggtaagctaa tgggacttcc tcacagattg aatgcgggag atgaggaaaa
86521 gagaaaaata caggctgtct cctatgtctt tggccagatt aactgggtag agtgagaaga
86581 ctggagaaca ctaagtttgt gaaaatctcc agatttcact ttgccaagtg tggtggcgca
86641 tgcctgtaat cccagctatg tgggaggctg aggcaggagg atcgcttggg cccaggaatt
```

```
86701 tgaggagttt gggattgcag tgatcatgcc actgcactcc agtctgggca acggagcaag

                                      *
                                      *
                                      *

88861 atccagtgac agagttcatg tggatttctt gttaaattct aactgcagag ctctaacttt
88921 tccctctaag ctcctgagag gcagattggc agctagtttc tcgaagaggt ttctgacagc
88981 cctgcattgg gtgatttcat tgaagggctt attttaagtt ctgagtcctc ctcccccatt
89041 cccccacatt agcattttca gccatgggtt gtggtgttaa ggacagggct gtatacgtgc
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89161 gaattcacag tgtggcttta ccgtgctgtc tggggcaaca taggtaagct ttaatgagcc
89221 ttagtttcct tatctaaggg aatatggaat taatatcaac cttaaagaac tgtttaaaat
89281 tctaaataaa tatttttata acatatgcta cttgaaggca aaaacaaggc cagtttatct
89341 tagtctacac ccaatacagg tggaaaatct aacatatttt tgaaggggtg ctctgttgag
89401 tttattaacc aagaaatgct aaactaatga caaaacatca ccttcagaag accaaaatca
89461 aaagttttac tacataaaga aaaaaagcac ctttgactct atttataaat ctgactttta
89521 aaaatgacca aaggaactat aatgtgaaac ccataaaccc aagcttgttt caaaatacat
89581 taaaaaaaat acttactcct ccacttgccc catgaaccag aacactctct ccagctttca
89641 cacaggcact gcaaaggaaa gcataagtta catcacctta ttttttgaag ctaattaatc
89701 tcgggtgttt tcatcatctt aaggaatttc tacccctagt ctggctaaca cttacacaaa
89761 cagcaaatgc aacctgacat acagccccaa atattcccta agctccacag aataaacaaa
89821 gccttcaatt catttattcc ttgaacaaat atttattggg agtctttatg ttccaggcac
89881 tatgctgctg gacactggga tgactatgtg gtgctacttc tgagtggcta cagtccttgt
89941 gggttgtgaa gtaaaattgc tgagcctgga ggatctggaa tctctcattc ccatatatcc
90001 cccacagaaa gggcctcaaa gcaggtttat tatatagctc agtctttatt ctgtggtcta
90061 gagtaatgtc caagtaaaca cagtagctat ttttttgcc caaggaaaga aagaaatttt
90121 tcttctccat gtctctgaac atcaggttgc accagccttg tactctttca gggaggaatg
90181 ctgagttagc aaaggtcaga gagtaggaaa tgcaataaat tctatcacaa agattcccat
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90361 gatgcctcac tattccagat ggacggtgcc aatgtcaaaa gccagcagat gctgtgagat
90421 ccagatctga ctctcaggaa ggctctctta cttcctcaaa caatgtgggg tggccacact
90481 gcagagacat tatagaacat tatgctccac ctgggaaaga gaacagtaac cagagtcctg
90541 ctcccagcta tgcaccaaca gctgagaagt ggcaacaatg agcaataagt gaagctttct
90601 cccacactct tgcttagagc tgaagggact gaggacaata tgttaaagta aaacataaac
90661 ataaggggat aggatgacta gtgttaaact atgggatatg aaatacctcc caaagaaatt
90721 tttcaaaaat tcttataaga tgcccctcaa acactaaaga cacattctca taaatccctg
90781 gggcctgggg tgaggggaga aaaagcaggc aaatcccctc ctgaatcctt gcacagagtc
90841 gctgtgacag ttaatttttat gtgtcaactt gactgggcca aggaacccaa tatttgttcc
90901 aacattactc tgttacagaa acagtgtttt ttttttttt cgaatgagat taacaatgga
90961 atagctggat tttgagtaaa gcagatgacc ctctagaatg tgggtgggcc tcatccaatc
91021 agttgaaggc ttttgttttc aaagactgac ctccgatgag caagagtaaa ttcagccagc
91081 aaactttcta tggacttaaa ctgcacctct tccttgtgtc tcccatctgc tggcccaccg
91141 caacagattt tagactcacc agtcctccac aatttcatgg gtcaactctt taaaatcaat
91201 caatctgtgt gcgcgtgtgt gtgtgtgtgt gtgtatgtgt acagagtgac tgattcttaa
91261 ggaatttata tagagataaa tgatagatca gatcaaatag aagatcaaat agatagatga
91321 ttgactgata gatagacaga cagacacaca tcccgttgtt tgtttctctg gagaaccctg

                                      *
                                      *
                                      *

147841 acagacagag atagacagag gcagagtcag ggagaggcag agaaagaaag agaacaagaa
147901 agcttaaaga tagtccaaac gcaaagctgt ctttaaaaaa tgcatactct attactggca
147961 acaaagtttt ataatctata cattttatga accactaatc cttaatttat tcaagatcac
148021 aacaggggac tcatattata gagtcaagta aatatcatta ccaacatttt atttaacagt
```

148081 ttgtcctcct taattacatg gagaatgata tagtgactcc ttcatgcctt tttttctcct
148141 taacaagcca tatgcaggaa agtttccatg ctgcgcaaac ataaaagaaa gttatatttc
148201 attcctaana gaaaactgaa aagc

==========

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OUTPUT FROM PROGRAM

NUMBER OF JOINS 4

1. 80513.... 81472
2. 81911.... 82219
3. 85116.... 85265
4. 89595.... 89651

JOIN NUMBER ----- 1
Length of pair 959
Starting position of block 79813
Block length (700 + pairlength +800) 2459
Block ...
agtttggctagttttggcttcaaaattaattaaactgtatcaatgtattttgaagtgttaagtcatctgtatgcttt
agctccttctatagatgaggcaaatatacaaacagattaaactgacttttacagaataattattcttttaccttgtt
tacatggaaaggaatcctccatttaggatgcacataaaatgccagcctatgttgatgacattgccttaacactttt
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cctgaagcaggattccatattcaagccagaaattgtcattcaacagaaaaaatcaggtcaaaacaatcaatcacata
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gccaaccaataatgaagtaaatattctaatatttgggaaaatactgagaaaactaataaattgtcctggatattatt
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caaagtccaatacagtcatggctaatcagagactagagaacctttataaaggtaagtaggcttgaaaacccttggaa
actgagcagtcttatttttgaactagcatgttttaatcaaaggtatggaattaatcaaatatcaattaagaattactg
gaatgcacactcatgccaaatgacaactaacatgttatttcctactatgatgactctttgatttgagtcagatggca
taaaaaaatattgctagctatacaataaattttactcttctgcttctgctctctaaagaaaaatcttatttttcac
ataagaagctcatggaatcgaatgttaattaaagaaaagatagggtaagtacaactgggggaaagacagtacctcta
attacataggaaatccatgaaagaattaatcatcataagagaagaatcatttttccagtagccccactaccatgaat
gatattttcatgagcctcggccaccttctccaatggatattgagaacctatcacaggtttcaaccagccaatttcca
ttccagcttgaagggctgctgcatattgctgaaattcctcctaagaaaaggaaaaacaaatttctttttgtagtgaa
ccgtatgatttaattttcagaagcattaaaaacacttcagaatctaagtgttataccatgaagagtctcttacaaat
gtgtgacttttgtcaacttgtccagaactatagaaaaagtagttatctacagggtaaccataaatcccatctgcctg
agacagtgttagtgtacaaaatacctgttgtcctgaaattattactagtatcacatttctatctcaaaaggtatgct
tacctggatataaattatactgtcaccctagttgtccttctggtgactaatccttaccaactcccactagtcatata
actaagtttaacatctattcaaactttcagcttgcctgagtaggcaaactgtaccaatgtttaagttaccaaaatca
gaagtacttcttttcctaccttggttgaggaaaagagagtaactccaattatactcgactcctttgccatggtgtct
cgtgggtttatttcaatagtacctctgctgccaacaacctaacatgaaaaacagcaattctacagttaaagattact
gtaaaatagtgttaaattgtggtaaaacattaaagtggtaaaaaaaaaaaaaagaaaaggaatacttactatcactc
gtcctccatgtgacagaagactcaagtctttactaagatttacattagctaacatttcaataattatatcaattcct
ttctcaccaacatacttctatataataaaagagaaatgtagagtaagatagcaagtgaaaaactgtaaaatag□

Actual comp position 80450 sequence tatgcagaagtgatgagaggc

Reverse comp position 80450 sequence gcctctcatcacttctgcata
g c t a toalno totalvalue 8 2 4 7 21 62

Actual comp position 81019 sequence tactggaatgcacactcatgc
Reverse comp position 81019 sequence gcatgagtgtgcattccagta
g c t a toalno totalvalue 4 6 5 6 21 62

JOIN NUMBER ----- 2
Length of pair 308
Starting position of block 81211
Block length (700 + pairlength +800) 1808
Block ...
tggaatcgaatgttaattaaagaaaagatagggtaagtacaactgggggaaagacagtacctctaattacataggaa
atccatgaaagaattaatcatcataagagaagaatcatttttccagtagccccactaccatgaatgatattttcatg
agcctcggccaccttctccaatggatattgagaacctatcacaggtttcaaccagccaatttccattccagcttgaa
gggctgctgcatattgctgaaattcctcctaagaaaaggaaaaacaaatttctttttgtagtgaaccgtatgattta
attttcagaagcattaaaaacacttcagaatctaagtgttataccatgaagagtctcttacaaatgtgtgacttttg
tcaacttgtccagaactatagaaaaagtagttatctacagggtaaccataaatcccatctgcctgagacagtgttag
tgtacaaaatacctgttgtcctgaaattattactagtatcacatttctatctcaaaaggtatgcttacctggatata
aattatactgtcaccctagttgtccttctggtgactaatccttaccaactcccactagtcatataactaagtttaac
atctattcaaactttcagcttgcctgagtaggcaaactgtaccaatgtttaagttaccaaaatcagaagtacttctt
ttcctaccttggttgaggaaaagagagtaactccaattatactcgactcctttgccatggtgtctcgtgggtttatt
tcaatagtacctctgctgccaacaacctaacatgaaaaacagcaattctacagttaaagattactgtaaaatagtgt
taaattgtggtaaaacattaaagtggtaaaaaaaaaaaaaaagaaaaggaatacttactatcactcgtcctccatgtg
acagaagactcaagtctttactaagatttacattagctaacatttcaataattatatcaattccttttctcaccaaca
tacttctatataataaaagagaaatgtagagtaagatagcaagtgaaaaactgtaaaatagctactatctgtacaag
atattatagaaatatgtttcaaatgatatataaatgctacatctttgagactaataatgcaaaattttaaataatct
aattatataatcacgatgtaattccaaggtaccagccagaacatctaaactgataaaaatttgtactaaatacattg
ctgtagtgaaataaagtttgtctggaattttcaggtgctagactcaacttgagtataaaatacttagctgaaaattt
tctatctgtaaaataaactttcataaagaaacaataaatcaaaagccccaaaccccccaggggctcccatttttatt
aataaacaaaaagcaaaagaagatatcattagctgttcggttttgcatgatttttgttgttttagtgcatttggttt
tgttctaaatggtttatcatctgtttgatgcactaactcttttgggctcttggatgttggacgctggctcttacaaa
aagctacacacatctacattatattcattttattttaacacacacacacaaatgaatccctgtgcccgggattgcac
taggtaccaggaatacaaatacaaacatagggagctcaaaacaaaactagtgagaaagatgggaaatactacagtca
tagctataaagtaatgggctaagtaacacattagcagaaataaatcatagaatacagagaaaaaaggttaaggtttg
attgcctgccatggtcagataaagttccacagagacga

Actual comp position 81844 sequence gcttgcctgagtaggcaaac
Reverse comp position 81844 sequence gtttgcctactcaggcaagc
g c t a toalno totalvalue 6 5 4 5 20 62

Actual comp position 82362 sequence tgtaattccaaggtaccagcc
Reverse comp position 82362 sequence ggctggtaccttggaattaca
g c t a toalno totalvalue 4 6 5 6 21 62

JOIN NUMBER ----- 3
Length of pair 149
Starting position of block 84416
Block length (700 + pairlength +800) 1649
Block ...

tgattgcaaaaaaggtaataaactctcactcttatttttccttcatttgtaatgatctaatttacacagtactcaa
tatttgggaaattctaatctccccaacgtgaggaagtggttgaggattagcaaagcaataagtgtttagcaaattgc
taatatagtacaagtgaagaacttcagaatctgcttgaattctgttaaatgcagcaactaaataaatgccacctcac
cattttggatgcagtagtgattattcctccaaagcatccagctaacaaatgaactttattccctgggccacacagat
ccagtttgtaatttacagatatctcaccttccatggagaattcacatcagtagaaattatattaagaatacctcaca
gctgcaaatacaaagctgcagctttacttagaatgttatttgcattaaaaaatcaatttttatagctctaagattct
agagaagctatattctatttaatacacataaacaatacaaaaatgatagtaaaagtttaaaacttagacatctgttt
tttaaataaattaaagttttaaaacacgcataaaaattcatcgcactgaaaaaaggaagcaaacagctttaaaggag
tagttggttaaaaacatattaaaaaaccacgcaagtctccaaggaacaaagtttgacttttgtaaaacagtggaaaa
ttttaccttaattttatcaatgtaattcacttctctgtgattgaacacttcatgggctccattttgcaaaacaatct
tttgtccttcctcagtaccagcagtgcccaaaatctttaagccataagctctagcaatttggcatgctgctaatcca
acctgaaaaacaaatataacccaagagttatatattctctacactcctgtaaacacttaaatacatacaatgaactt
aagattcctataggacccaccctaactttaaggaacttaagagtgtaaatgaagaaataagaaaaacagctaacttt
aattgagcatttaaaatattccaggaaccatactaaataatttctacatattgttttattctatcctcacaatgacc
ctataaagtagatactattattgtccctattgtacagataagaaagttgaagcttcaaattataagtaatttggcca
agtcatatgcggagatggaaacaggagttagaccagtctgactgcagaacttgagtttttaaccactgcatcaagat
gtttgcagggtttaaagatgatcagaacatgctctctgacttctttgtgcatatgaaattctaaataacaaatgtaa
ggcctccaccatttaagtagaagagataggtatatgggcaaattaactaattcatccatatggtgaatgtttataga
gtgtttacgatgtgctagacatggtacttaatgtaagaaataaacttatattctaagggtggaggaagataatagtc
atatgaatgaataaaataaattcaggaaataaaagtgctaagaaaaaataagactggctgttgggttaaagagacag
gaataggggctatttaggtcatcaggaagagccactctgaaaaaatgagacctgaaaaaagtgaggaacaagccacg
agaacatccggtcagccacgtggaggatgctgt□

Actual comp position 85062 sequence gcaagtctccaaggaacaaag
Reverse comp position 85062 sequence ctttgttccttggagacttgc
g c t a toalno totalvalue 5 5 2 9 21 62

Actual comp position 85563 sequence gatggaaacaggagttagacc
Reverse comp position 85563 sequence ggtctaactcctgtttccatc
g c t a toalno totalvalue 7 3 3 8 21 62

JOIN NUMBER ----- 4
Length of pair 56
Starting position of block 88895
Block length (700 + pairlength +800) 1556
Block ...
attctaactgcagagctctaacttttccctctaagctcctgagaggcagattggcagctagtttctcgaagaggttt
ctgacagccctgcattgggtgatttcattgaagggcttattttaagttctgagtcctcctcccccattcccccacat
tagcattttcagccatgggttgtggtgttaaggacagggctgtatacgtgcactccatggatgtcatcaaagtgcag
caggcaagcagcagaagggagatagaaggactaagaattcacagtgtggctttaccgtgctgtctggggcaacatag
gtaagctttaatgagccttagtttccttatctaagggaatatggaattaatatcaaccttaaagaactgtttaaaat
tctaaataaatatttttataacatatgctacttgaaggcaaaaacaaggccagtttatcttagtctacacccaatac
aggtggaaaatctaacatatttttgaaggggtgctctgttgagtttattaaccaagaaatgctaaactaatgacaaa
acatcaccttcagaagaccaaaatcaaaagttttactacataaagaaaaaaagcacctttgactctatttataaatc
tgacttttaaaaatgaccaaaggaactataatgtgaaacccataaacccaagcttgtttcaaaatacattaaaaaaa
atacttactcctccacttgccccatgaaccagaacactctctccagctttcacacaggcactgcaaaggaaagcata
agttacatcaccttatttttgaagctaattaatctcgggtgttttcatcatcttaaggaatttctacccctagtct
ggctaacacttacacaaacagcaaatgcaacctgacatacagccccaaatattccctaagctccacagaataaacaa
agccttcaattcatttattccttgaacaaatatttattgggagtctttatgttccaggcactatgctgctggacact
gggatgactatgtggtgctacttctgagtggctacagtccttgtgggttgtgaagtaaaattgctgagcctggagga
tctggaatctctcattcccatatatcccccacagaaagggcctcaaagcaggtttattatatagctcagtctttatt
ctgtggtctagagtaatgtccaagtaaacacagtagctatttttttgcccaaggaaagaaagaaatttttcttctc
catgtctctgaacatcaggttgcaccagccttgtactctttcagggaggaatgctgagttagcaaaggtcagagagt
aggaaatgcaataaattctatcacaaagattcccatgtcatccccctgaaatgtccagattctctggtgaaatggca

```
ttttctttttacttccagttcacatgactacttttctagtatgtactgaaaagaagggacatgcagcaaggcatgag
gggatgcctcactattccagatggacggtgccaatgtcaaaagccagcagatgctgtgagatccagatctgactctc
aggaaggctctcttact□

Actual comp position    89543    sequence    gtgaaacccataaacccaagc
Reverse comp position   89543    sequence    gcttgggtttatgggtttcac
 g c t a toalno  totalvalue 3     7  2  9  21  62

Actual comp position    90103    sequence    ctccatgtctctgaacatcag
Reverse comp position 90103    sequence    ctgatgttcagagacatggag
 g c t a toalno  totalvalue 3  7  6  5  21  62
```

==========

An additional rule relating to gene family members may also be included in the set of primer selection rules. Many genes in the human genome are members of gene families, which means that they closely resemble other genes at other positions in the genome. When primer sequences are selected for a certain gene, one may later find that the selected primers are actually undesirably present in these other family members. The cycle of selecting an appropriate primer sequence for a given gene, that is, identifying a candidate primer sequence, searching the public database to find out whether or not it is specific to that gene, identifying that it is not specific to the gene, reselecting another candidate primer sequence, etc., could go on for several loops before an appropriate primer sequence is identified.

An example command for operating the function for this task is:

```
primer611 sult1a1.txt sult1a1join.txt primerout sult1a2.txt sult1a3.txt
```

where the program executable command is primer611, the input sequence file within which to find primers is sult1a1.txt, the input join file that tells the program where the

coding (exons) regions is sult1a1join.txt , the output file is primerout, and the other two files, sult1a2.txt and sult1a3.txt, are sequence files of family members. The number of gene family files which may be included can be large.

When the program selects a candidate primer in the sult1a1.txt file, it then reads the sult1a2.txt and sult1a3.txt files to see if it is present. If it is present, it discards it and selects another candidate primer. If it is not present in the files, it selects and stores it and goes on to find the next primer. The program also looks at the family member files in both forward and reverse directions to be complete and eliminate the user from having to format these files to be in the proper coding orientation.

Thus, the software can select primers that are unique to the gene of interest and can be relied upon for genes that are members of families. This functionality can be added to the functionality of picking the best primers around the exons of a gene for the primer design process -- select the candidate primer only if it is unique to the target file and not present in the gene family files.

To further illustrate the functionality and output, below is a listing of the primeronly file and and a portion of the primerout file (listing the 1st three primer pairs). The command used to generate this output is:

```
primer611 topo2a.txt topo2ajoin.txt primerout topo2b.txt chr18.txt.
```

The primerout file is defined in the fourth element of the above command and the primeronly file below is created and named automatically. The primerout file has

each of the exon regions defined in the topo2ajoin.txt file printed out with "....." before and after the exon, and documents the steps that the program went through when picking the primers. The primerout file lists candidate primer sequences that otherwise met the primer selection rules, but was found in one of the gene family files and was therefore rejected (see areas that read "FOUND in"). The output presentation allows a user to go back to a specific region and redesign a primer if the primer selected happens to be in a repetitive sequence region not screened out with the gene family files. This may be done, for example, by doing a database search.

```
================================================================================
"PRIMERONLY" FILE
================================================================================

topE1E2-5   actgtggaaacagccagtaga
□
topE1E2-3   tcttgataacctcgctgtgtc
□

□
topE3E4E5-5
□
topE3E4E5-3
□

□
topE6E7E8-5 atgtgccaccctctatccag
topE6E7E8-3 ttagagatgatgaataaagctcc

topE9E10E11-5     cccagcctaacagttcttttg
topE9E10E11-3     ccactacgctcggccaattt

topE12E13E14-5    aagagaacagtaactcccgtc
topE12E13E14-3    cagcactgattccatgcatac

topE15-5    gccagaagttgtaggttcaag
topE15-3    ctttactcagtcccaagctct

topE16-5    gcgtgacacatagcaagtgc
topE16-3    gccagttcttcaatagtaccc

topE17E18E19-5    gagaagaacctttgccaatgg
topE17E18E19-3    ctccaccattactctcaccaa

topE20E21E22-5    tgcctgtataccgggatatac
```

```
topE20E21E22-3    ttgacaaaggtatactgctgga

topE23-5    cttctgtctccacaccttcc
topE23-3    ggagaggtgagagagagatg

topE24-5
topE24-3

topE25E26E27-5    aattgtttctcctactaccctc
topE25E26E27-3    aacccatctcaaagatttaggc

topE28E29-5 aatgcctgtattgaattgcagg
topE28E29-3 taaaaccagtcttgggcttgg

================================================================================


================================================================================
"PRIMEROUT" FILE
================================================================================

Gene Name        :    top


Sequence File    :    topo2a.txt
 Join File           :    top2ajoin.txt
 Output File         :    primerout

 No of Family sequence files:    2
 Family Sequence File:    topo2b.txt
 Family Sequence File:    chr18.txt
 Number of characters in Sequence file            :    22080
 Number of Lines in Sequence file                 :    2

JOIN Values .....             29

1    1         66        topE1
2    290       502       topE2
3    1443      1616      topE3
4    1806      1907      topE4
5    2015      2152      topE5
6    4630      4768      topE6
7    5136      5293      topE7
8    5586      5711      topE8
9    6318      6428      topE9
10   6571      6676      topE10
11   6767      6876      topE11
12   8378      8470      topE12
13   8770      8884      topE13
14   8988      9109      topE14
15   10207     10355     topE15
16   12180     12411     topE16
17   12598     12732     topE17
18   12852     13052     topE18
19   13194     13389     topE19
20   14138     14229     topE20
```

```
    21    14332      14496      topE21
    22    14628      14711      topE22
    23    16803      16934      topE23
    24    18702      18854      topE24
    25    19098      19221      topE25
    26    19328      19371      topE26
    27    19799      19933      topE27
    28    21275      21474      topE28
    29    21792      22080      topE29

 SORTED JOIN Values .....

    1     1          66         topE1
    2     290        502        topE2
    3     1443       1616       topE3
    4     1806       1907       topE4
    5     2015       2152       topE5
    6     4630       4768       topE6
    7     5136       5293       topE7
    8     5586       5711       topE8
    9     6318       6428       topE9
    10    6571       6676       topE10
    11    6767       6876       topE11
    12    8378       8470       topE12
    13    8770       8884       topE13
    14    8988       9109       topE14
    15    10207      10355      topE15
    16    12180      12411      topE16
    17    12598      12732      topE17
    18    12852      13052      topE18
    19    13194      13389      topE19
    20    14138      14229      topE20
    21    14332      14496      topE21
    22    14628      14711      topE22
    23    16803      16934      topE23
    24    18702      18854      topE24
    25    19098      19221      topE25
    26    19328      19371      topE26
    27    19799      19933      topE27
    28    21275      21474      topE28
    29    21792      22080      topE29


 COMBINED JOIN Values .....

    1     1          502        topE1E2
    2     1443       2152       topE3E4E5
    3     4630       5711       topE6E7E8
    4     6318       6876       topE9E10E11
    5     8378       9109       topE12E13E14
    6     10207      10355      topE15
    7     12180      12411      topE16
    8     12598      13389      topE17E18E19
    9     14138      14711      topE20E21E22
    10    16803      16934      topE23
    11    18702      18854      topE24
    12    19098      19933      topE25E26E27
```

```
13   21275     22080     topE28E29

 Total no of joins :    13


 PAIR NO :    1         First     1         Second    502       Name
topE1E2
 PAIR Length .....            501

 Block Length .....          :    1301

 Block starting position..... :    0

n .....
nnnattcagtaccaaatttactgtggaaacagccagtagagaatacaagaaaatgttcaaacaggcaagtaaataag
tgtcttgtaccttaatgataaatggtagtagtatagccatttataatggcattaatgattggtttaatttaacataa
tttataagctattgaagtatggaaaattataagcatatatattaggttattaggactcataaatttatgttatttac
ttccagtttgtgagatgacttgaatttttcatgtttcctattctttacttccatagacatggatggataatatggga
agagctggtgagatggaactcaagcccttcaatggagaagattatacatgtatcacctttcagcctgatttgtctaa
gtttaaaatgcaaagcctggacaaagatattgttgcactaatggtcagaagagcatatgatattgctggatccacca
aagatgtcaaagtctttcttaatggaaataaactgccat .....
gagtattttcctggatgttaaggataataagggattttgtaatcattgtcaagtgcaaaattgaatttttccccctc
ccatatgtttttgtttgtttgtttgtttgtttgtttgagacagagtctcacactgttgcccgggctggagtgcagtg
gcacgatctcggctcaccgcaacctccacctcccaggttcacgcaattctcctgcctcagcctcccaagtagctggg
attacaggtgcctgccaccacacctggctaatttttgtatttttagtagagacaggtttcactatgttggccaggc
tggtctcgaacaccagacctcatgatccacccgtcttggcctcccaaagtgctgggattacaggcatgagccactgc
acctggcccaaccatatgtattttcttaccacttctcacatatgttcttgaaaagagaatggtatgccacatttttt
aatcagctcatttaaacttaccgaaggaatttctttctcaaagaaacacctaaaataaatatttcatgtccttttt
ttatttttcctttttctttctttttcttgataacctcgctgtgtcacccaggctggagtacagtgatgcaatcacggct
cactacagcctggacctcccaggctcaagcgatcatcccacctcagcttctggagtagctggaaatgcaggcagcac
caccatgcccagctaatttttttttttctttttaatagaggtggggatctcactatgttgcccaggctggtcttgaa
ctcctgggctcaagtgatccacccacctc□
 Did not get PRIMER , what to do , DO NOT HAVE ENOUGH CHARACTERS:    1  TO
DEAL
Seq ..    tcttgataacctcgctgtgtc  FOUND  in  :  chr18.txt at  8964   position
Seq ..    ttgataacctcgctgtgtcac  FOUND  in  :  chr18.txt at  8966   position
Seq ..    gataacctcgctgtgtcacc  FOUND  in  :  chr18.txt at  8968   position
Seq ..    ataacctcgctgtgtcaccc  FOUND  in  :  chr18.txt at  8969   position
Seq ..    caggctggagtacagtgatg  FOUND  in  :  chr18.txt at  8988   position
Seq ..    aggctggagtacagtgatgc  FOUND  in  :  chr18.txt at  8989   position
Seq ..    ctggagtacagtgatgcaatc  FOUND  in  :  chr18.txt at  8992   position
Seq ..    ggagtacagtgatgcaatcac  FOUND  in  :  chr18.txt at  8994   position
Seq ..    gagtacagtgatgcaatcacg  FOUND  in  :  chr18.txt at  8995   position
Seq ..    agtacagtgatgcaatcacgg  FOUND  in  :  chr18.txt at  8996   position
Seq ..    cagtgatgcaatcacggctc  FOUND  in  :  chr18.txt at  9000   position
Seq ..    gtgatgcaatcacggctcac  FOUND  in  :  chr18.txt at  9002   position
Seq ..    gcaatcacggctcactacag  FOUND  in  :  chr18.txt at  9007   position
Seq ..    caatcacggctcactacagc  FOUND  in  :  chr18.txt at  9008   position
Seq ..    aatcacggctcactacagcc  FOUND  in  :  chr18.txt at  9009   position
Seq ..    tcaagcgatcatcccacctc  FOUND  in  :  chr18.txt at  9043   position
Seq ..    aagcgatcatcccacctcag  FOUND  in  :  chr18.txt at  9045   position
Seq ..    gatcatcccacctcagcttc  FOUND  in  :  chr18.txt at  9049   position
Seq ..    tcatcccacctcagcttctg  FOUND  in  :  chr18.txt at  9051   position
Seq ..    cacctcagcttctggagtag  FOUND  in  :  chr18.txt at  9057   position
Seq ..    acctcagcttctggagtagc  FOUND  in  :  chr18.txt at  9058   position
Seq ..    ctcagcttctggagtagctg  FOUND  in  :  chr18.txt at  9060   position
```

```
Seq ..    tcagcttctggagtagctgg  FOUND  in  :  chr18.txt at  9061   position
Seq ..    cttctggagtagctggaaatg  FOUND  in  :  chr18.txt at  9065   position
Seq ..    ttctggagtagctggaaatgc  FOUND  in  :  chr18.txt at  9066   position
Seq ..    ggagtagctggaaatgcagg  FOUND  in  :  chr18.txt at  9070   position
Seq ..    gagtagctggaaatgcaggc  FOUND  in  :  chr18.txt at  9071   position
Seq ..    gtagctggaaatgcaggcag  FOUND  in  :  chr18.txt at  9073   position
Seq ..    tagctggaaatgcaggcagc  FOUND  in  :  chr18.txt at  9074   position
Seq ..    gggatctcactatgttgccc  FOUND  in  :  chr18.txt at  9139   position

 PRIMER 2 actual              :    -2130704935 ... tctcactatgttgcccaggc

Letters    20 g count    4 t count    6 c count    7 a count    3 total
62

 reverse                     :    -2130704935 ... gcctgggcaacatagtgaga
          topE1E2-3     gcctgggcaacatagtgaga

 Number of letters between pairs:    -2131274831


 PAIR NO :    2         First     1443      Second    2152      Name
topE3E4E5
 PAIR Length .....            709

 Block Length .....          :    2208

 Block starting position..... :    743

tgcctgccaccacacctggctaatttttttgtatttttagtagagacaggtttcactatgttggccaggctggtctcg
aacaccagacctcatgatccacccgtcttggcctcccaaagtgctgggattacaggcatgagccactgcacctggcc
caaccatatgtatttttcttaccacttctcacatatgttcttgaaaagagaatggtatgccacatttttttaatcagct
cattttaaacttaccgaaggaatttctttctcaaagaaacacctaaaataaatatttcatgtcctttttttatttttc
ctttttctttctttttcttgataacctcgctgtgtcacccaggctggagtacagtgatgcaatcacggctcactacag
cctggacctcccaggctcaagcgatcatcccacctcagcttctggagtagctggaaatgcaggcagcaccaccatgc
ccagctaatttttttttttttctttttaatagaggtggggatctcactatgttgcccaggctggtcttgaactcctggg
ctcaagtgatccacccacctcggcctgtgtcctttaatgaccattcccttatgcctatcagtgaacatcattgcatt
ggttttggaaagtcctcatagtctatcattgaacctatttttttaataactttcttaatactgttacctttaattcct
gtacagg .....
aaaaggatttcgtagttatgtggacatgtatttgaaggacaagttggatgaaactggtaactccttgaaagtaatac
atgaacaagtaaaccacaggtgggaagtgtgtttaactatgagtgaaaaaggctttcagcaaattagctttgtcaac
agcattgctacatccaaggtaatttttattcttaaattattaatcatgatttatctttacatatatgtgttcttattg
tttttaatatataaagtggacttgaatattgggctagcttagtataaaggaggttaaattagtttttaatgtttgat
tattataatttttgaggatactgagttttacagtttggtatttttccttattagggtggcagacatgttgattatgta
gctgatcagattgtgactaaacttgttgatgttgtgaagaagaagaacaagggtggtgttgcagtaaaagcacatca
ggtatgtgcttttggcagttttcttttttctaaagtcaaggaagaagagaaaggctataaataaagcatgagtacatt
tttagtggcttaatatcaacttctattgcaggtgaaaaatcacatgtggatttttgtaaatgccttaattgaaaacc
caacctttgactctcagacaaaagaaaacatgactttacaacccaagagctttggatcaacatgccaattgagtgaa
aaatttatcaaagctt .....
gagtacttagaggaaaataaaaatagaaacacctgactttattttccattgcacttcttagctctgcagaaacaatg
attcttctcatagtgagcttctccaagtcttcccaatctgaaaaggaagtaaaaaagggctttacttttaactgattt
accaaagacttaatgaccgtctatatttcagtatttcccaattacattttaccattaagcttagatcacttttgaat
taatctagctgtttaacaaacaccctcacttaaatgcctaagacttgctttcagtcaacacatccaaaattgaattt
gttacctccatactcactgatttgcccatacaagcagccccccactctccaacaaaaaaacaacttcctatcttagt
aaaaagccccaaccaacctctaggttgtataaacaagaaagctgggagccttcctttatttcccctcctctctaatc
cggtcaataagaatcatctcttggatgctgcagtagcttctcaccattatctctttttggtttactacaataggtt
cttaaccttcatactggttaagtcctttccttggaatgcttttgagtgacttttgtgttaaaacacccatttttatc
```

```
ttcactctcatttgaaatctttcaatgacttccactcagggaaagtccaaattccataatttggccaacaagaaaga
tctgctgtaatctaattacacctacttctccaactcatctcagtgccagtttttcgtatattgtcctgttgctttta
aattactgaaaagcacagtgctcttcccc□Seq ..    ccattcccttatgcctatcag  FOUND  in  :
chr18.txt at  9221   position
Seq ..    gaccattcccttatgcctatc  FOUND  in  :  chr18.txt at  9219   position
Seq ..    tcaagtgatccacccacctc  FOUND  in  :  chr18.txt at  9182   position
Seq ..    actcctgggctcaagtgatc  FOUND  in  :  chr18.txt at  9172   position
Seq ..    tgaactcctgggctcaagtg  FOUND  in  :  chr18.txt at  9169   position
Seq ..    cttgaactcctgggctcaag  FOUND  in  :  chr18.txt at  9167   position
Seq ..    aggctggtcttgaactcctg  FOUND  in  :  topo2b.txt at  36055   position

 PRIMER 1:    1246 ... tcactatgttgcccaggctg

Letters    20 g count    5 t count    6 c count    6 a count    3 total
62
          topE3E4E5-5     tcactatgttgcccaggctg
Seq ..    gcctaagacttgctttcagtc  FOUND  in  :  chr18.txt at  10319   position
Seq ..    cctccatactcactgatttgc  FOUND  in  :  chr18.txt at  10365   position
Seq ..    ctccatactcactgatttgcc  FOUND  in  :  chr18.txt at  10366   position
Seq ..    tccatactcactgatttgccc  FOUND  in  :  chr18.txt at  10367   position
Seq ..    cactgatttgcccatacaagc  FOUND  in  :  chr18.txt at  10375   position
Seq ..    ctgatttgcccatacaagcag  FOUND  in  :  chr18.txt at  10377   position
Seq ..    tgatttgcccatacaagcagc  FOUND  in  :  chr18.txt at  10378   position
Seq ..    tttgcccatacaagcagccc  FOUND  in  :  chr18.txt at  10381   position
Seq ..    cccaaccaacctctaggttg  FOUND  in  :  chr18.txt at  10445   position
Seq ..    taaacaagaaagctgggagcc  FOUND  in  :  chr18.txt at  10467   position
Seq ..    caagaaagctgggagccttc  FOUND  in  :  chr18.txt at  10471   position
Seq ..    aagaaagctgggagccttcc  FOUND  in  :  chr18.txt at  10472   position
Seq ..    ctgggagccttcctttatttc  FOUND  in  :  chr18.txt at  10479   position
Seq ..    tgggagccttcctttatttcc  FOUND  in  :  chr18.txt at  10480   position
Seq ..    gaatcatctcttggatgctgc  FOUND  in  :  chr18.txt at  10525   position
Seq ..    atcatctcttggatgctgcag  FOUND  in  :  chr18.txt at  10527   position
Seq ..    atctcttggatgctgcagtag  FOUND  in  :  chr18.txt at  10530   position
Seq ..    ctcttggatgctgcagtagc  FOUND  in  :  chr18.txt at  10532   position
Seq ..    ggatgctgcagtagcttctc  FOUND  in  :  chr18.txt at  10537   position
Seq ..    tgctgcagtagcttctcacc  FOUND  in  :  chr18.txt at  10540   position
Seq ..    ctggttaagtcctttccttgg  FOUND  in  :  chr18.txt at  10605   position
Seq ..    ttcaatgacttccactcaggg  FOUND  in  :  chr18.txt at  10689   position
Seq ..    atgacttccactcagggaaag  FOUND  in  :  chr18.txt at  10693   position
Seq ..    cttccactcagggaaagtcc  FOUND  in  :  chr18.txt at  10697   position
Seq ..    ctcagggaaagtccaaattcc  FOUND  in  :  chr18.txt at  10703   position
Seq ..    tggccaacaagaaagatctgc  FOUND  in  :  chr18.txt at  10730   position
Seq ..    gccaacaagaaagatctgctg  FOUND  in  :  chr18.txt at  10732   position
Seq ..    cacctacttctccaactcatc  FOUND  in  :  chr18.txt at  10764   position
Seq ..    cctacttctccaactcatctc  FOUND  in  :  chr18.txt at  10766   position
Seq ..    cttctccaactcatctcagtg  FOUND  in  :  chr18.txt at  10770   position
Seq ..    ttctccaactcatctcagtgc  FOUND  in  :  chr18.txt at  10771   position
Seq ..    ctccaactcatctcagtgcc  FOUND  in  :  chr18.txt at  10773   position
Seq ..    ccaactcatctcagtgccag  FOUND  in  :  chr18.txt at  10775   position

 Did not get PRIMER , what to do , DO NOT HAVE ENOUGH CHARACTERS:    2208  TO
DEAL

 PAIR NO :    3        First     4630      Second    5711      Name
topE6E7E8
 PAIR Length .....           1081
```

Block Length ..... : 2580

Block starting position..... : 3930

gatctcagttcactgcaacccgcgcctcccaggttaaagcaattctcctgcctcagcctcccaagcagctaggatta
cagccatctcaccaccaccatgcctggctacccttttttttttttttttttttttgagacggagtttcactttt
gtcacccaggctggagtgcaatggtgcgatcttggctcgctgcaacctctacctcctgggttcaagcgattctcctg
cctcagcctcccgagtagctggaattacaggtgcccaccaccacgccagctaatttttgtatttttagtagagccgg
ggtttcgccatgttggccaggccggtctcaaactcctgacctcaggtgttctgcccaccttggcctcctaaagtgct
gggattataggcgtgagccaccgtgcctggtctaatttgttttaaccactatatctccaacaagtagctcagtgcta
gcacaatataattatatagtaaatatttattgaacgaatgaaccaaaaggagcagctccctcagtggtgataacctg
acatgggaagatgtgccaccctctatccagaaattattgttctacatcttttaatttttgaatcatttttatttgt
attaaggctcatttgtattctagatttctgatagatcccttcttccctaatatgatccctaatatgaatcttctcgt
tttcagg .....
cattggctgtggtattgtagaaagcatactaaactgggtgaagtttaaggcccaagtccagttaaacaagaagtgtt
cagctgtaaaacataatagaatcaagggaattcccaaactcgatgatgccaatgatgcaggtatatatttaataatg
tttccaaacttttaagtcttatagttgttattttattcattaatggcataccacggatatttatttttcccttgaca
gaataactatattcaacagaataacttgttaaaaatcggcccgtttcctattatggaagatttaggtcatttccatg
ttataaataatattgaggtgattattttggagtataaaacaagaatgtttatattatgatctattacctaacaaata
attttgctcattatatagtaaattgtgttttatcacaaggctataaacagcatgttcaagttagtatatttgaggtt
gaactaaatgtgctaatattaatatgtatatttttatttaggggccgaaactccactgagtgtacgcttatcctg
actgagggagattcagccaaaactttggctgtttcaggccttggtgtggttgggagagacaaatatggggttttccc
tcttagaggaaaaatactcaatgttcgagaagcttctcataagcaggtagaatataagacgatcttcagaatctaaa
tctaatttataatacaagactttatgcttatatttaattccctcattaggcattttaaaatatattttagacaattt
gtgcttatttgagaaattaggtacattgtagcctattttaacagacctttctgatgtagtaaattataagctaata
gctcaaaatactggagctcaagaaaatccaagcaacatatactgttaaatttctttgttcttttcaaatttataaac
gatgctttttttggtatatgtccatttcagatcatggaaaatgctgagattaacaatatcatcaagattgtgggtct
tcagtacaagaaaaactatgaagatgaagattcattgaagacgcttcgttatgggaagataatgattatgacagatc
agt .....
cagatttgttattaaatttttagattgttcaactaaattaagcatgtcttaatttaatttcattgtttttgccatg
aaaataaattacttaaataggagctttattcatcatctctaatcaacatctaatcagatatgcttatatcatatgta
tgttgcaaatacaggttaagtgagtctggatttgaacagacctttttttgattcccatagaaaatttgacaaattgcc
agtaggtcagtcataatatttttttatttctaaacaattctttgtttgtttgagatggagtttcgcccttgtcgccc
aggctggagtgcaatggtgcaatcttggctcactgcaacctccgcctcatgggttcaagcgattctcctgcctcagc
ctcccgagtagctgggattgcaggcggatgccaccacacccaactaatttttgtatttttagtggagacagggtttc
accatgttggccaggctggtctcgaacgcctgacctcaggcgatccgcctgcctcggcctcccaaagttctgggatt
acagatgttagctaccacgcccagcctaacagttcttttgaactttggctttcaaatctttctaggaccaagatggt
tcccacatcaaaggcttgctgattaattttatccatcacaactggccctctcttctgcgacatcgttttctggagga
atttatcactcccattgtaaaggtacgctaatttctaagtaccatcatggatattttaagaccctactcctcaaacc
tggatatacatataagccccgtcacatgt

PRIMER 1: 4479 ... atgtgccaccctctatccag

Letters 20 g count 3 t count 5 c count 8 a count 4 total
62

topE6E7E8-5 atgtgccaccctctatccag

PRIMER 2 actual : 6005 ... gagtgcaatggtgcaatcttg

Letters 21 g count 7 t count 6 c count 3 a count 5 total
62

reverse : 6005 ... caagattgcaccattgcactc

topE6E7E8-3 caagattgcaccattgcactc

Number of letters between pairs: 1526

*
*
*

==========

There are two gene family files in this comparison. The topo2b.txt file is a human genome sequence for a gene called topoisomerase 2b, which is highly related to the gene of interest, topoisomerase 2a. In the primerout file, many of the candidate primers the program selected were present in this family member and were therefore rejected. This demonstrates the utility of the functionality of this program. The second family member sits on chromosome 18 and is a pseudogene (a duplicated region of DNA that does not make a real gene -- a serious nuisance for designing primers that are to amplify a single genetic position). The program has accommodated for this as well; it selected a candidate primer that was found in this file a large number of times.

Without this functionality, primers that would amplify three different regions at the same time would be designed: the topo2a region of interest; the topo2b region related to it; and a nuisance region in chromosome 18. Unfortunately, the resulting data would show numerous discrepancies that are not real polymorphisms. These sequences are actually from different genetic positions that are highly similar to one another but not identical. Thus, most of the "SNPs" found in this manner are not SNPs at all. If one tried to genotype people at a "false SNP," they would get incoherent data as they would be looking at three different positions within the genome at the same

time. It is important to produce data for single positions at a time so that the data can be accurately read and interpreted.

Advantageously, the rules that the inventive software uses in the preamplification process are different than those of conventional programs in that they are suitable for use in designing high throughput experiments where many different things can be done simultaneously. It is more efficient to do simultaneous amplifications of four or five regions in 500 people, for example, rather than doing them one by one. This is where the rule regarding the fixed predetermined annealing temperature (e.g., 62° Celsius) comes into play: since all of the primers selected by the program have the same annealing temperature, the work can be done more efficiently. Another example is where the software automatically decides if a single primer pair can be utilized for two or more coding regions, which saves additional time and expense. Furthermore, the rule regarding gene family data is important for generating reliable output data and for efficiency.

The output of the software is also unique. The numbers included in the output use the numbering pattern that exists in the input sequence file (for example, starting at "10003") rather than starting at "1" like most other programs. This means that a primer at position "11234" can be quickly located, whereas in other programs the number for the primer would be "1231" and one would have to perform the math to figure out its location. This is particularly important for those primers that have to be redesigned manually due to having certain characteristics that can only be determined through a database search.

Additional Details Regarding The Discovery of Reliable SNP and Haplotype Data. The description that follows provides additional details regarding steps 318-342 of FIG. 3B, which may be referred to as part of the post-amplification process. As described earlier, one important goal of the program is to find reliable discrepancies between individuals at a sequence of a particular genetic locus or location in the genome. To do this, the inventive methods use a direct measure of the nucleotide base quality, or "phred" score, of an observed discrepancy (at steps 326-328 of FIG. 3B).

Actual DNA sequence data files, called chromatograms, are utilized as input, as quality information is an inherent part of such files. As is well-known, a sequence chromatogram looks like a series of colorful peaks and valleys. The color of a peak indicates the DNA base present at that position in the sequence. Peaks in a graph for a good sequence tend to be higher than for a bad sequence, and overlapping peaks tend to indicate poor reliability. Such information is used to determine whether a discrepancy in a sequence alignment represents a good candidate SNP or not.

The functionality of a conventional phred program is used to call the quality of every letter, and the program aligns the sequences and finds where they are "reliably" different from one another. By reliable, it is meant that the differences in sequence are differences between letters of good quality. An example of one such program is the phred program available from the University of Washington, which ascribes a numerical value to indicate the quality of each letter of a sequence. The phred functionality makes a separate file with all of these numbers, for each letter.

DNA sequences from various individuals are aligned using a conventional sequence alignment algorithm (at step 320), such as that provided using conventional Clustal software functions available by and from the EMBL, Heidelberg Germany, and is a re-write of the popular Clustal V program described by Higgins, Bleasby, and Fuchs (1991) CABIOS, 8, 189-191 (Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) (CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Research, 22:4673-4680). Thus, the sequence alignment file is the first input file to the program. Any discrepancy that occurs within a neighborhood of other discrepancies is recognized so that the quality value information can be checked. If this information is greater than predetermined quality information, such as a user-defined input value, it is accepted and presented to the user for final acceptance. If not, it is discarded. The quality control file created from the phred functionality serves as the second input file.

In the sequence within which the discrepancy occurs, positions of the minor letters of the discrepancy are presented to the end-user. This lets the end-user contemporaneously call up the raw DNA sequence chromatogram and find the actual trace data peak for the letter. This is advantageous because a visual inspection of raw DNA sequence data is the most reliable method of determining whether a discrepancy is valid. While the purpose of the software is to eliminate many time consuming steps, in some cases, borderline quality values nonetheless necessitate its execution. The presentation of the precise position and relevant file names for a discrepancy makes this

step easy to execute. Also, the end-user is shown presentations of discrepancies that do not meet the quality control criteria. This is important because, in some cases, a borderline quality value may conceal good data due to other problems with sequence compressions or peak spacing.

Another important attribute is afforded the software because it can recognize reliable base deletion polymorphisms. This is performed by parsing the phred quality data for the bases surrounding the deletion in randomly selected sequences which contain the deletion. With conventional programs, if a discrepancy is a deleted base there is no quality control information to check since no data is produced for a non-base (and there is consequently no phred value for the deleted base). This eliminates any discovery of single base deletion polymorphisms. Deletion polymorphisms are common and, since the goal is to thoroughly document the various genetic haplotypes in a population, a SNP-finding program that can recognize deletion polymorphisms offers competitive advantages. Not knowing all of the variants in a gene sequence causes the resolution of haplotype-based studies to be sub-optimal, compared to being able to recognize all variants (including deletion polymorphisms).

The software may also incorporate rules to maximize efficiency during these steps. For example, the program may focus on determining the phred value for discrepancies that fall within a block of sequence with an acceptable average phred value. As another example, the user-defined phred value could be different for different regions of the sequence. In another variation, the program is configured to recognize amino acid differences by translating the sequences and instructed to only

present candidate polymorphisms that result in a change in amino acid sequence.

Example Walk-Through. Input = (1) Clustal W alignment file and (2) phred quality file. The user inputs a minor letter phred quality control value for the current run, as well as a local phred quality control value. For example, the user may enter the values "24" and "17" for the the minor letter and local phred quality control values, respectively. Then, from the first input file, each column (position or slice) of the alignment is analyzed to determine whether the column is homogeneous (i.e., whether each sequence has the same letter at that position) or heterogeneous (i.e. whether there are two or more different letters at that position).

As an example, consider the following:

```
AHRE11-3          AGGGGGTAGATTTTAAAAAT-CATGTTAATGTTATTTACT-
AHRE11-3-E10      AGGGGGTAGATTTTAAAAAT-CATGTTAATGTTATTTACT-
AHRE11-3a         AGGTGTAAGATTTTAAAAATACATGTTAATGTTATTTACT-
AHRE11-3u         AGGGGTA-GATTTCAAAAATACATGTTAATGTTATTTACT-
14                AGGGGTA-GATTTTAAAAATACATGTTAATGTTATTTACT-
AHRE11-3-C4       AGGGGTAAGATTTTAAAAATACATGTTAATGTTATTTACT-
AHRE11-3-D5       AGGGGTAAGATTTTAAAAATACATGTTAATGTTATTTACT-
```

The first column of letters is homogeneous. So is the second and third. The fourth is heterogeneous, as is the sixth, etc.

The second input file is the phred quality file, which takes the format of the 1XN matrix below for each sequence. The entry for the first sequence above (AHRE11-3) appears below:

```
>AHRE11-3 folder=AHRE11-3 length=414
8 9 23 24 32 34 27 27 34 34 32 32 34 34 32 32 29 29 26 26 26 28 34 31 29 29
32 35 35 35 45 45 45 40 35 35 39 32 33 32
```

In this file, the first two letters are of very low quality or reliability because, for biochemical reasons, sequencing reactions routinely have trouble at the beginning of a sequence read.

For each column of the alignment, the software recognize whether there is a discrepancy (i.e., major and minor letters.) If a discrepancy exists, then the following logic is executed:

```
      For each minor letter, read the phred value.
For example, in column 14 above, sequence AHRE11-3u
has a C but the others have a T.  The "C" is a minor
letter and it has the value 34.
      Calculate the average phred value for the major
letter (G in column 14 above)
      Calculate the average phred value for each
minor letter (in column 14 above, there is only one
minor so this is the same as the phred value for that
letter.
      Determine the number of major letters.
      Determine the number of minor letters.
      Calculate the average phred value for the block
of letters 7 in front and 7 behind the column using
all of the input sequences and their quality values.
This will be called the local phred quality value.
```

To process the job, the phred value of the minor letter and average phred value of the major letter are utilized such that

```
      If the phred value of any minor letter in the
column is greater than the user-defined threshold
value,
      And
      If the average phred value of the major letter
for the column is above a different threshold value
defined by the user,
      Then label the column as accepted and present
to the user for visual inspection.
```

Alternatively, a more sophisticated method for determining the worth of a positional column is to use a function to calculate the probability that a column contains

a reliable polymorphism using the average quality value for the column, the quality values for the minor letters, the quality value for the region around the column (using all the sequences), or other variables. For this approach the following logic is utilized:

1) A column with a high average major letter phred score and a high minor letter phred score is a better column than one with
a) a low average major letter phred score and a high minor letter phred score;
b) a high average major letter phred score and a low minor letter phred score;
c) a low average major letter phred score and a low minor letter phred score; and
2) A column with a discrepancy in a region of sequence that has a high local phred quality value is better than one in a region with a low local phred quality value.

Preferably, a probability function is employed for this task, including variables for that which is measured above. For example, one might use Bayes' theorem to calculate this probability; for every column a vector is created from the variables calculated above and the linear equation:

$y=A_1X_1+A_2X_2+A_3X_3...A_nX_n$
giving the vector $Y=(A_1,A_2,A_3...A_n)$, where An are parameters.
Then determine a Bayesian estimate
$p(w|x) = [p(x|w)p(w)]$ divided by $p(x)$,
where $p(w|x)$ = classification score of the column as good or bad or somewhere in between (called the posterior probability), $p(x)$ is the frequency or uniqueness or worth of this vector, and $p(w)$ is the frequency or uniqueness of the class. $P(x|w)$ is the conditional probability that x is observed given that w is also observed - in this frequency that vectors of the above An are observed for true SNP columns (determined using other suitable biochemical techniques).

Once the alignment file has been inspected for every column, the results are presented to the user. For example, if the probability is high that a column contains a reliable polymorphism, then the column is presented to the user along with 7 letters in front and 7 letters behind for each sequence in the alignment. For example,

```
Sequence 1  TTTATCTGACTGGAG
Sequence 2  TTTATCTGACTGGAG
Sequence 3  TTTATCTCACTGGAG
```

Also, the "average" sequence 200 letters in front and 200 letters behind the column is presented. For example,

```
ATTATGCTCG  ATTATGCTCG  ATTATGCTCG  ATTATGCTCG  ATTATGCTCG
ATTATGCTCG  ATTATGCTCG  ATTATGCTCG  ATTATGCTCG  ATTATGCTCG
ATTATGCTCG  ATTATGCTCG  ATTATGCTCG  ATTATGCTCG  ATTATGCTCG
ATTATGCTCG  ATTATGCTCG  ATTATGCTCG  ATTATGCTCG  ATTATGCTCG  G/C
ATTATGCTCG  ATTATGCTCG  ATTATGCTCG  ATTATGCTCG  ATTATGCTCG
ATTATGCTCG  ATTATGCTCG  ATTATGCTCG  ATTATGCTCG  ATTATGCTCG
ATTATGCTCG  ATTATGCTCG  ATTATGCTCG  ATTATGCTCG  ATTATGCTCG
ATTATGCTCG  ATTATGCTCG  ATTATGCTCG  ATTATGCTCG  ATTATGCTCG
```

In the above example, there is only one column with discrepancies; each of the other columns are homogeneous. In practice, this will be unusual and the presentation will look more like the following (note the letters R, Y, M):

```
YTTATGCTCG  ATTATGCTCG  ATTATGCTCG  ATTATGCTCG  ATTATGCTCG
ATTATGCTCG  ATTATGCTCG  RTTATGCTCG  ATTATGCTCG  ATTATGCTCG
ATTATGCTCG  ATTATGCTCG  ATTATGCTCG  ATTATGCTCG  ATTATGCTCG
ATTATGCTCG  ATTATGCTCG  ATTATGCTCG  ATTATGCTCG  ATTATGCTCG  S
ATTATGCTCG  ATMATGCTCG  ATTATGCTCG  ATTATGCTCG  ATTATGCTCG
ATTATGCTCG  ATTATGCTCG  ATTATGCTCG  ATTATGCTCG  ATTATGCTCG
ATTATGCTCG  ATTATGCTCG  ATTATGCTCG  ATTATGCTCG  ATTATGCTCG
ATTATGCTCG  ATTATGCTCG  ATTATGCTCG  ATTATGCTCG  ATTATGCTCG
```

Where

R=A or G

```
Y=C or T
K=G or T
M=A or C
S=G or C
W=A or T
N=any base
B=C,G, or T
D=A,G or T
H=A,C or T
V=A,C or G
```

Other information may also be presented, such as the following: (a) for each sequence with a minor letter, the sequence name and the associated phred value for the minor letter; and (b) the local region phred score.

Example Output. Below is a file that shows what the software produces as it inspects a single discrepancy.

```
================================================================================

k = 70
Position of Reference sequence without dashes :
65
Position of complement sequence: 209

Indicator          ^


QUALITY INFORMATION
      Discrepancies at position
70

Minor letter 1::-::1
Minor letter 2::A::1
Major letter ::G::60
 Got '-' as minor value

 Got 1
minor characters
 Minor characters ::: A

      Check quality for mlnor A

Got sequence , sequence
id AHRE9-5-D7
No of dashes before minor
character position      67
Quality value (
4) is lessthan24 at position 4
Total No of minor charaters quality is less than24 is 1
```

Total No of minor charaters
quality is greater than24 is 0

AHRE9-5-D2 C-TCTGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-H1 C-TCTGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-C4 C-TTTGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-B5 C-TCTGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-D5 C-TTTGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-A6 C-TCTGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-B2 C-TCTGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-C3 C-TCTGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-C2 C-TCTGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-D3 C-TCTGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-E2 C-TTTGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-F2 C-TCTGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-E1 C-TCTGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-G2 C-TCTGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-G3 C-TCTGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-H2 C-TTTGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-D1 C-TTTGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-F1 C-TTTGAGTTA;ACcumulated SNP # : Q S
AHRE9-5-D12 CATTCGAGTTA;Accumulated SNP # : O S
AHRE9-5-B4 CAT-CGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-D6 CAT-CGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-C1 CAT-CGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-A12 CAT-CGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-B11 CAT-AGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-D7 --AATAGAGTA;Accumulated SNP # : 1 S
AHRE9-5-H12 ------GGTTA;Accumulated SNP # : 0 S
AHRE9-5-D4 C-TCTGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-C5 C-TCTGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-B1 C-TCTGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-B3 C-TCTGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-A3 C-TCTGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-C6 CAT-CGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-F11 C-TCCGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-G11 C-TCCGAGTTA;Accumulated SNP # : O S
AHRE9-5-C12 C-TTCGAGTTA;Accumulated SNP # : O S
AHRE9-5-E10 C-TCCGAGTTA;AcCumulated SNP # : O S
AHRE9-5-C10 CTC-CGAGTTA;Accumulated SNP # : O S
AHRE9-5-G12 CTCNCGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-D10 CATTCGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-D8 CATTCGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-D9 CATCCGAGTTA;Accumulated SNP # : O S
AHRE9-5-E11 C-TCCGAGTTA;Accumulated SNP # : O S
AHRE9-5-C9 CAT-TGAGTTA;Accumulated SNP # : O S
AHRE9-5-E8 TATTCGAGTTA;Accumulated SNP # : O S
AHRE9-5-B10 TCATCGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-D11 TCTTCGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-C8 CAT-CGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-B8 TCTTCGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-F8 TCTCNGAGTTA;Accumulated SNP # : O S
AHRE9-5-H11 TCTCCGAGTTA;Accumulated SNP # : O S
AHRE9-5-A8 CAT-CGAGTTA;Accumulated SNP # : O S
AHRE9-5-F12 C-TTCGAGTTA;Accumulated SNP # : O S
AHRE9-5-E12 C-TCCGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-F7 CATCCGAGTTA;Accumulated SNP # : 0 S

```
AHRE9-5-G10 C-TCCGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-B9  C-TTCGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-C7  --CTTGAGT-A;Accumulated SNP # : 0 S
AHRE9-5-F10 AATCCGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-C11 CATTCGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-A10 ACTCCGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-F9  C-TCCGAGTTA;Accumulated SNP # : 0 S
AHRE9-5-G8  C-TCCGAGTTA;Accumulated SNP # : 0 S

Left  :
Right :
AGTTACAATGATATAATCTGGTCTTCCATTTTTATAAAGCAGGCGTGCATTAGACTGGACCCAAGTCCATCG
GTTGTTTTTTGTAAGAAGCCGGA-
AAACTATCATGCCACTTTCTCCANTCTTAATCACTAAAATAAAATTAAAWA---
ATTAAATTATCAAACCCCCAAATC-AATATAGTAAAGATTATTCCTAAAA

Do you want to choose this into SNP data ?[y/n] n
*********************************************************************

=====================================================================
```

Now consider the text window below which shows an alignment produced by the software. Note the small numbers at the end of most of the lines (most are 0, some 1; one 17, one 22). When a discrepancy in the last two sequences having a quality score on the borderline is seen, and the number of "Accumulated SNPs" is high as it is shown in the last two lines, the discrepancy can be ignored as the large number indicates that the sequence is of poor quality. This inference is good because real SNPs occur at a frequency of about 1 in 200 letters and the high numbers are much greater than one would expect. If it were not for these numbers, one would have to go and look at the sequence trace file to see if the discrepancy was real or not. Using this technique, it has never been observed that a discrepancy in a sequence with a large Accumulated SNP number turns out to be a real SNP upon visual inspection of the trace data. Thus, time can be saved by avoiding to have to regularly view such trace data.

```
=====================================================================
S13462.DPG-51-CP1 ACAATCCTTAA;Accumulated SNP # : 0 S
S13462.DPG-90-CP1 ACAATCCTTAA;Accumulated SNP # : 0 S
```

```
S13462.DPG-92-CP1 ACAATCCTTAA;Accumulated SNP # : 0 S
S13462.DPG-83-CP1 ACAATCCTTAA;Accumulated SNP # : 0 S
S13462.DPG-75-CP1 ACAATCCTTAA;Accumulated SNP # : 0 S
S13462.DPG-22-CP1 ACAATCCTTAA;Accumulated SNP # : 0 S
S13462.DPG-37-CP1 ACAATCCTTAA;Accumulated SNP # : 1 S
S13462.DPG-96-CP1 ACAATCCTTAA;Accumulated SNP # : 1 S
S13462.DPG-93-CP1 ACAATCCTTAA;Accumulated SNP # : 1 S
S13462.DPG-12-CP1 ACAATCCTTAA;Accumulated SNP # : 1 S
S13462.DPG-20-CP1 ACAATCCTTAA;Accumulated SNP # : 0 S
S13462.DPG-59-CP1 ACAATCCTTAA;Accumulated SNP # : 0 S
S13462.DPG-86-CP1 ACAATCCTTAA;Accumulated SNP # : 0 S
S13462.DPG-16-CP1 ACAATCCTTAA;Accumulated SNP # : 1 S
S13462.DPG-19-CP1 ACAATCCT--A-;Accumulated SNP # : 1 S
S13462.DPG-42-CP1 ACAAACCT-----;Accumulated SNP # : 17 S
S13462.DPG-14-CP1 ACAAACCTTAT;Accumulated SNP # : 22 S
Indicator ^
mar 204 404
Right Margin
Left :
CTCAGGTCCCACAGCAACAATATCATTCAAACTGCAATTAAAACATACACACATAATATATAAGGTGAAGGT
ATTGAACATTACAGGATTATTAACTGGCATTCCTCACTGTCTATTCCTAAAATCAAGATGTGGGATGGAGCCTTCGT
GCT
AGCTATAATGGAACACAATTAATATGAAATTAGTCCTGCCGATACAAT
Right : CTTAAAGGGCGAATTCGTTTAAACCTGCAGGACTAG--------------------------------
---
---
Quality Values for Minor :::
18
Total No of minor charaters quality is less than 21 is 1
Total No of minor charaters quality is greater than 21 is 0
Do you want to choose this into SNP data ?[y/n]
================================================================================
```

The inventive software has several useful features which distinguish it from other programs that use phred quality control data to find reliable discrepancies:

1) Other phred-based programs simply present the discrepancies that show a phred value above some arbitrary number. The problem is that it is quite common to find discrepancies with letters having quality values. Take the example below:

```
TAATTC
ATAATT
TAATTC
TAATTC
```

Note that the second sequence is "shifted" relative to the other three due to one single sequencing mistake called an insertion, which is common. The alignment program is not perfect and does not always make the correct alignment by shifting the sequences relative to one another. Even though the quality values for the letters A, T, A, A, T and T are very good, they are not SNPs but rather sequencing/alignment errors. Most other programs would output these letters as good candidate SNPs, so if the end-user did not go back to the data to inspect it valuable time and expense would be incurred by designing genotyping experiments based on incorrect data.

The inventive program avoids this by visually presenting a local neighborhood of sequences to the end-user for those discrepancies that meet the phred threshold value. In other words, the program presents a block of sequences (such as the one above) so that an experienced user can recognize common errors such as this shift error.

Other common errors the end-user might notice are discrepancies in strings of sequence (such as GGGGG), or a phenomena called "bleedthrough". A conventional program relying just on phred score would select those mistakes and bad experiments would subsequently be designed. Since the inventive program shows the local sequence around this region for all the sequences, it is obvious to a trained molecular biologist that the finding by the software is incorrect and should be discarded.

So one advantage of the software is that it presents a snapshot of the data, along with a query line asking if the user wishes to accept the data or not, so that invaluable human input is included in the SNP discovery analysis.

2) Another advantage is that the precise position and sequence that the discrepancy occurs is readily apparent to the user. The example output above shows how this data is presented. Notice that each discrepancy is advantageously identified by using k = "column number". This is important in case the end-user wants to call up the sequence data electropherogram, since it tells him which one to call up and where to go to see the relevant base. This is often done in different windows on the desktop. Visual inspection of raw DNA sequence data is the most reliable method of determining whether a discrepancy is valid. While the purpose of software is to eliminate such time consuming steps, in some cases borderline quality values require visual inspection. The presentation of the precise position and relevant file names for a discrepancy makes this step easy to perform.

3) Another advantage is that the end-user can specify a quality control value for a run of the program, then go back and repeat the run using a different quality control value. The quality for a position that meets the threshold requirements is also reported to the user so that borderline cases can be further reviewed.

4) Yet even another advantage is that the program presents the neighboring 200 letters of average sequence (for all of the individuals in an analysis) in front of and behind candidate SNP locations. This is important because when submitting SNP locations to a SNP consumables company (e.g., Orchid), one must submit the neighboring sequence as well so that the kit can be designed to assay this SNP in thousands of people.

5) Finally, another advantage is that the user can visualize deletion mutations, which do not have corresponding phred values. A unique attribute is afforded the software because of this functionality. The program can recognize reliable base deletion polymorphisms and present them to the user for visual inspection. In conventional programs, if a discrepancy is a deleted base there is no quality control information to check since no data is produced for a non-base or deleted base (and there is consequently no phred value for the deleted base). This would eliminate the discovery of single base deletion polymorphisms. Deletion polymorphisms are common and, since the goal is to thoroughly document the various genetic haplotypes in a population, a SNP finding program that can recognize deletion polymorphisms offers competitive advantages. Not knowing all of the variants in a gene sequence causes the resolution of haplotype-based studies to be sub-optimal, compared to being able to recognize all of the variants.

In an alternate embodiment, the software does not use actual DNA sequence data files or chromatograms but rather accepts and utilizes sequence information in text format which is freely available and downloadable from publicly available databases. For quality control, an indirect measure of quality is used. For example, any discrepancy that occurs within a bleedthrough region, or within the neighborhood of discrepancy clusters is ignored.

It should be readily apparent and understood that the foregoing description is only illustrative of the invention and in particular provides preferred embodiments thereof. Various alternatives and modifications can be devised by those skilled in the

art without departing from the true spirit and scope of the invention. For example, gene data from human, animal, plant, or other may be utilized in connection with the methods. Accordingly, the present invention is intended to embrace all such alternatives, modifications, and variations which fall within the scope of the appended claims.

What is claimed is: